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## **ABSTRACT:**

To examine tumor angiogenesis in the tumor microenvironment, we studied malignant ascites and tumors of patients with untreated ovarian carcinoma. We observed that malignant ascites fluid induced potent in vivo neovascularization in Matrigel assay. We detected sizable amount of VEGF in malignant ascites. However, pathological concentration of VEGF is insufficient to induce in vivo angiogenesis. Ovarian tumors SDF-1/CXCL12 is released into malignant ascites. High concentration of CXCL12, but not the pathological concentration of CXCL12 induces in vivo angiogenesis. Strikingly, pathological concentrations of VEGF and CXCL12 efficiently and synergistically induce in vivo angiogenesis. We show that VEGF upregulates the receptor for CXCL12, CXCR4 expression on vascular endothelial cells (VECs), and synergizes CXCL12-mediated migration of VECs. Further, CXCL12 synergizes VEGF-mediated VEC expansion, and synergistically protects VECs from sera starvation-induced apoptosis with VEGF. Finally, we show that hypoxia synchronously induces CXCL12 and VEGF production. Therefore, hypoxia-induced signal would be the important factor for initiating and maintaining an active synergistic angiogenic pathway mediated by CXCL12 and VEGF. Altogether, these results demonstrate that hypoxia triggered tumor CXCL12 and VEGF form a synergistic angiogenic axis in vivo. Interrupting this synergistic axis, rather than VEGF alone, will be a novel efficient anti-angiogenesis strategy to treat cancer.

## Table of Contents

Cover.....	
SF 298.....	
Table of Contents.....	4
Introduction.....	5
Body.....	5
Key Research Accomplishments.....	6
Reportable Outcomes.....	7
Conclusions.....	7
References.....	8
Appendices.....	8

## **INTRODUCTION:**

Ovarian carcinoma is the fifth leading cause of cancer among women and leading cause of mortality among cancers of the female reproductive system (1). Ovarian carcinomas have a poor prognosis, often associated with multifocal intraperitoneal dissemination with potent neovascularization. The related mechanism remains poorly understood.

One of the most well characterized angiogenic factors is vascular endothelial growth factor (VEGF). VEGF has angiogenic action in numerous in vivo and in vitro models (2, 3). Many anti-angiogenic strategies have targeted VEGF activity. Some reports of tumor regression in experimental models of angiogenesis exist. The majority of studies show anti-angiogenic therapy leads to an inhibition of tumor growth rather than a regression of established tumors (4, 5). Early clinical trials with anti-angiogenic strategies, however, have not replicated the results observed from preclinical models (2, 6, 7).

On the other hand, CXCR4, the receptor for CXCL12, appears to mediate the progression and metastasis of cancers in mice (8). The current explanation is that anti-CXCR4 blocks CXCR4 expressing tumor migration to CXCL12 expressing tissues (8-11). More strikingly, recent reports (12-14) suggest that certain angiogenesis inhibitors (or antagonists) alone, by depriving tumors of oxygen, could have an unintended effect: promotion of tumor metastasis by increasing CXCR4 expression. Ovarian tumor cells produce a large amount of CXCL-12 (15), and release into peritoneal cavity. We thus studied the role and regulation of tumor derived CXCL-12 and VEGF in human ovarian cancer.

## **BODY:**

Ovarian carcinomas have a poor prognosis, often associated with multifocal intraperitoneal dissemination accompanied by intense neovascularization. To examine tumor angiogenesis in the tumor microenvironment, we studied malignant ascites and tumors of patients with untreated ovarian carcinoma.

The research materials and experimental methods are published in our recent article in "Materials and Methods" section (page 465-466) (Kryczek et al, Cancer Res, 65:465-472, 2005).

We observed that malignant ascites fluid induced potent in vivo neovascularization in Matrigel assay (Fig 1A) (Kryczek et al, Cancer Res, 65:465-472, 2005). Vascular endothelial cell growth factor (VEGF) is one of the most important angiogenic factors. We detected sizable amount of VEGF (3.1 ng/ml) in malignant ascites (page 466, text) (Kryczek et al, Cancer Res, 65:465-472, 2005). However, pathological concentration of VEGF is insufficient to induce in vivo angiogenesis in our Matrigel model (Fig 3A, 3B, page 468) (Kryczek et al, Cancer Res, 65:465-472, 2005). We now show that ovarian tumors strongly express CXC chemokine stromal-derived factor (SDF-1/CXCL-12) (Fig 2, page 467) (Kryczek et al, Cancer Res, 65:465-472, 2005). Tumor derived CXCL-12 is released into malignant ascites (25 ng/ml). High concentration of CXCL-12 (> 50 ng/ml), but not the pathological concentration of CXCL-12 induces in vivo angiogenesis (Fig 3, page 468) (Kryczek et al, Cancer Res, 65:465-472, 2005). Strikingly, pathological concentrations of VEGF and CXCL-12 efficiently and synergistically induce in vivo angiogenesis (Fig 3, page 468) (Kryczek et al, Cancer Res, 65:465-472, 2005). Migration, expansion and survival of vascular endothelial cells form the essential functional network of angiogenesis. We further provide a mechanistic basis for explaining the interaction between CXCL-12 and VEGF.

We show that VEGF upregulates the receptor for CXCL-12, CXCR4 expression on vascular endothelial cells, and synergizes CXCL-12-mediated migration of vascular endothelial cells. Further, CXCL-12 synergizes VEGF-mediated vascular endothelial cell expansion, and synergistically protects vascular endothelial cells from sera starvation-induced apoptosis with VEGF (Fig 4, page 469) (Kryczek et al, Cancer Res, 65:465-472, 2005). After determining that CXCL12 and VEGF form a synergistic angiogenic pathway, we next examined the potential regulatory mechanisms for the production of CXCL12 and VEGF. We showed that 4-6 hours after exposure to hypoxia, the level of VEGF165 and VEGF121 mRNA was 50 and 2.5 fold higher, respectively, in hypoxia-treated tumor cells than normoxia-treated tumor cells (Fig 5A) (page 470, Kryczek et al, Cancer Res, 65:465-472, 2005). As confirmation, hypoxia-treated ovarian tumor cells released more VEGF protein than normoxia ( $n = 8$ ,  $P < 0.001$ ) (Fig 5B) (page 470, Kryczek et al, Cancer Res, 65:465-472, 2005). Strikingly, 4 hours after exposure to hypoxia, CXCL12 mRNA was significantly induced ( $n = 12$ ) (Fig 5C). At 6 hours, the level of CXCL12 mRNA was 100 fold higher in hypoxia than normoxia ( $n = 12$ ) (Fig 5C) (page 470, Kryczek et al, Cancer Res, 65:465-472, 2005). We observed similar results in one commercialized ovarian tumor cell line (BG-1) and 3 primary ovarian tumors cell lines (OC8, OC21 and OC38) established in the laboratory. As confirmation, intracellular staining demonstrated that the level of CXCL12 protein was significantly higher in tumor cells exposed to hypoxia than normoxia ( $n = 8$ , \*,  $P < 0.001$ , compared to normoxia) (Fig 5D) (page 470, Kryczek et al, Cancer Res, 65:465-472, 2005).

Altogether, hypoxia activates the synergistic angiogenic pathway between VEGF and CXCL12 through synchronously triggering VEGF and CXCL12 production, and tumor derived CXCL-12 and VEGF form a synergistic angiogenic axis in vivo. Interrupting this synergistic axis, rather than VEGF alone, will be a novel efficient anti-angiogenesis strategy to treat cancer.

## **KEY RESEARCH ACCOMPLISHMENTS**

Tumor derived SDF-1 and VEGF synergistically induced angiogenesis.

SDF-1 and VEGF synergistically regulate CXCR4 expression.

Hypoxia regulates tumor derived SDF-1 and VEGF production.

Tumor associated PDC induced angiogenesis in vivo.

Tumor associated PDC IL-8 and TNF- $\alpha$  are the critical angiogenesis factors

Tumor associated MDC suppressed angiogenesis in vivo

Tumor associated MDC derived IL-12 is critical for suppressing angiogenesis in vivo.

## REPORTABLE OUTCOMES

Published manuscripts:

Weiping Zou. Immunosuppressive networks in the tumor environment and their therapeutic relevance. *Nature Review (Cancer)*, 2005, 5:263-274.

Kryczek I, Cheng P, Mottram P, Alvarez X, Moons L, Evdemon-Hogan M, Wei S, Zou L, Machelon V, Emilie D, Lackner A, Curiel TJ, Carmeliet P, Zou W. CXCL-12 and VEGF synergistically induce neoangiogenesis in human ovarian cancers. ***Cancer Res*, 2005, 65, 465-472.**

Zou L, Barnett B, Safah H, LaRussa VF, Evdemon-Hogan M, Mottram P, Wei S, David O, Curiel TJ, **Zou W.** Bone marrow is a reservoir for CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells that traffick through CXCL12/CXCR4 signals. ***Cancer Res* 2004, 64, 8451-8455.**

Curiel J, Cheng P, Mottram P, Alvarez X, Kryczek I, Moons L, Evdemon-Hogan M, Wei S, Zou L, Hoyle G, Lackner A, Carmeliet P, **Zou W.** Dendritic cell subsets differentially regulate angiogenesis in human ovarian cancer. ***Cancer Res* 2004, 64, 5535-5538.**

Curiel, T.J., G. Coukos, L. Zou, X. Alvarez, P. Cheng, P. Mottram, M. Evdemon-Hogan, J.R. Conejo-Garcia, L. Zhang, M. Burow, Y. Zhu, S. Wei, I. Kryczek, B. Daniel, A. Gordon, L. Myers, A. Lackner, M.L. Disis, K.L. Knutson, L. Chen, and **W. Zou.** Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. ***Nat Med* 2004,10:942-949.**

## CONCLUSIONS

In summary, our data demonstrate a novel role for SDF-1 and VEGF in human cancer. Tumor derived SDF-1 and VEGF synergistically induce tumor angiogenesis. This is a previously unappreciated angiogenesis pathway. Blocking SDF-1 and VEGF, rather than VEGF alone may be a novel strategy to block tumor neoangiogenesis.

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## APPENDICES

Included 5 original copy of journal articles

# CXCL12 and Vascular Endothelial Growth Factor Synergistically Induce Neoangiogenesis in Human Ovarian Cancers

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## Abstract

Ovarian carcinomas have a poor prognosis, often associated with multifocal i.p. dissemination accompanied by intense neovascularization. To examine tumor angiogenesis in the tumor microenvironment, we studied malignant ascites and tumors of patients with untreated ovarian carcinoma. We observed that malignant ascites fluid induced potent *in vivo* neovascularization in Matrigel assay. We detected a sizable amount of vascular endothelial cell growth factor (VEGF) in malignant ascites. However, pathologic concentration of VEGF is insufficient to induce *in vivo* angiogenesis. We show that ovarian tumors strongly express CXCL12. High concentration of CXCL12, but not the pathologic concentration of CXCL12 induces *in vivo* angiogenesis. Strikingly, pathologic concentrations of VEGF and CXCL12 efficiently and synergistically induce *in vivo* angiogenesis. Migration, expansion, and survival of vascular endothelial cells (VEC) form the essential functional network of angiogenesis. We further provide a mechanistic basis for explaining the interaction between CXCL12 and VEGF. We show that VEGF up-regulates the receptor for CXCL12, CXCR4 expression on VECs, and synergizes CXCL12-mediated VEC migration. CXCL12 synergizes VEGF-mediated VEC expansion and synergistically protects VECs from sera starvation-induced apoptosis with VEGF. Finally, we show that hypoxia synchronously induces tumor CXCL12 and VEGF production. Therefore, hypoxia triggered tumor CXCL12 and VEGF form a synergistic angiogenic axis *in vivo*. Hypoxia-induced signals would be the important factor for initiating and maintaining an active synergistic angiogenic pathway mediated by CXCL12 and VEGF. Thus, interrupting this synergistic axis, rather than VEGF alone, will be a novel efficient antiangiogenesis strategy to treat cancer. (Cancer Res 2005; 65(2): 465-72)

## Introduction

Tumor angiogenesis is essential for the growth of primary and metastatic tumors. Tumors and metastases may originate as small avascular masses that induce the development of new blood vessels once they grow to a few millimeters in size (1, 2). One of the most well characterized angiogenic factors is vascular endothelial cell growth

factor (VEGF). VEGF has angiogenic action in numerous *in vivo* and *in vitro* models (3, 4). Many antiangiogenic strategies have targeted VEGF activity. Some reports of tumor regression in experimental models of angiogenesis exist. The majority of studies show antiangiogenic therapy leads to an inhibition of tumor growth rather than a regression of established tumors (5, 6). Early clinical trials with antiangiogenic strategies, however, have not replicated the results observed from preclinical models (3, 7, 8). Previously identified angiogenic molecules  $\beta 3$  and  $\beta 5$  integrins have recently been shown not to support *in vivo* angiogenesis (9, 10). Angiogenic molecule basic fibroblast growth factor is found positively related to the prolonged survival of tumor patients (11). The reasons for these apparent discrepancies are that the extent of angiogenesis is determined by multiple factors in tumor microenvironment and each individual tumor may display a different angiogenic phenotype. Some other potential angiogenic factors may also have functionally been ignored in the designs of antiangiogenic strategies.

Ovarian carcinoma is the fifth leading cause of cancer among women and leading cause of mortality among cancers of the female reproductive system (12). Ovarian carcinomas have a poor prognosis, often associated with multifocal i.p. dissemination with potent neovascularization. The related mechanism remains poorly understood. Ovarian tumor cells produce a large amount of CXCL12 (13) and release into peritoneal cavity. In this report, we show that hypoxia importantly and synchronously induces CXCL12 and VEGF production by tumors, and CXCL12 and VEGF form a synergistic angiogenic axis to induce angiogenesis *in vivo*.

## Materials and Methods

**Human Subjects and Clinical Samples.** We studied patients with ovarian carcinomas. Patients are given written informed consent. The study was approved by the local Institutional Review Board. No cancer patients received prior specific treatments. Ascites have been collected from consecutive patients with previously untreated ovarian carcinoma. Ascites were collected aseptically, and harvested cells by centrifugation over a Ficoll-Hypaque density gradient (13).

***In vivo* Matrigel Assay.** We established an *in vivo* Matrigel assay in mice (14-17). Briefly, 0.5 mL of iced Matrigel (Becton Dickinson, San Jose, CA) admixed with the relevant cytokines or ascites and heparin were injected into the right lower abdomen of female C57 mice (6-8 weeks). After 10 to 12 days (16), the Matrigel plugs were isolated and processed for quantifying microvessel density (18) with ImagePro Plus software (Image-Pro plus, Media Cybernetics, Silver Spring, MD). Microvessel density was expressed as mean percentage of microvessel surface area by confocal Leica TCS-NT SP microscope.

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**Immunohistochemistry.** Matrigel plugs were subjected to immunohistochemistry analysis with rabbit anti-human-vWF antibody (polyclonal, 1/100 dilution, DAKO, Carpinteria, CA), and further stained with goat anti-rabbit antibody (immunoglobulin G, 1/2,000 dilution, Molecular Probes, Eugene, Oregon). Surface occupied by vascular endothelial cells (vWF<sup>+</sup> green cells) was quantified by confocal microscope as described above. Tumor tissues CXCL12 expression was analyzed by immunohistochemistry with 8- $\mu$ m cryosections of acetone-fixed ovarian tumor tissues as we described previously (13, 19). Tumor tissues were incubated for 2 hours at room temperature with anti-CXCL12 antibody (clone K15C, IgG2a, 10  $\mu$ g/mL), or control isotype. Antibody binding was detected with biotinylated anti-mouse antibodies and streptavidin conjugated to alkaline phosphatase (Biogenex, San Ramon, CA) using fast red substrate. Sections were counterstained with Mayer hematoxylin.

**Reverse Transcriptase-PCR.** CXCL12 and VEGF mRNA was detected by reverse transcriptase-PCR (RT-PCR) as we described (20). Briefly,  $\beta$ -actin was initially amplified and quantified with serial dilutions of cDNA from each sample. CXCL12 was then amplified in each sample containing identical amount of  $\beta$ -actin mRNA.  $\beta$ -Actin primers were sense 5'-gggtcagaaggattccatg-3' and antisense 5'-gggtctcaaacatgatctggg-3'. CXCL12 primers were sense 5'-gggtcctctgggtttgtatt-3' and antisense 5'-gtcctgagagtccttttgcg-3'. The identical technique and primers were used to amplify each VEGF splice forms as previously published (21).

**Migration Assay.** Fresh human umbilical vascular endothelial cells (HUVEC) were purified from human umbilical cords as we described (22). HUVECs were transferred into the upper chambers of 8- $\mu$ m-pore transwell plates (Neuro Probe, Gaithersburg, MD). CXCL12 and VEGF (R&D System, Minneapolis, MN) were added to the lower chamber. After 40 hours at 37°C, migration was quantified by counting cells in the lower chamber and cells adhering to the bottom of the membrane (13).

**Cell Proliferation.** Fresh HUVECs ( $10^5$ /mL) were cultured with the indicated cytokines for 72 hours.  $^3$ H thymidine was added at the last 16 hours, and cell proliferation was detected by thymidine incorporation as we described (13, 23, 24).

**In vitro Apoptosis Assay.** Fresh HUVECs ( $5 \times 10^5$ /mL) were cultured in 37°C with different concentrations of FCS in medium with the described conditions. After 24 hours, cells were harvested and stained with Annexin V and 7-AAD. Apoptosis of HUVEC was analyzed by fluorescence-activated cell sorting (13).

**Hypoxia Experiments.** Primary ovarian tumor cells ( $5 \times 10^5$ /mL or  $2 \times 10^6$ /mL) were cultured in 37°C incubators with 1% oxygen (Coy Laboratory Products, Inc., Grass Lake, MI) or 21% oxygen with the described conditions. Cells were harvested for detecting VEGF and CXCL12.

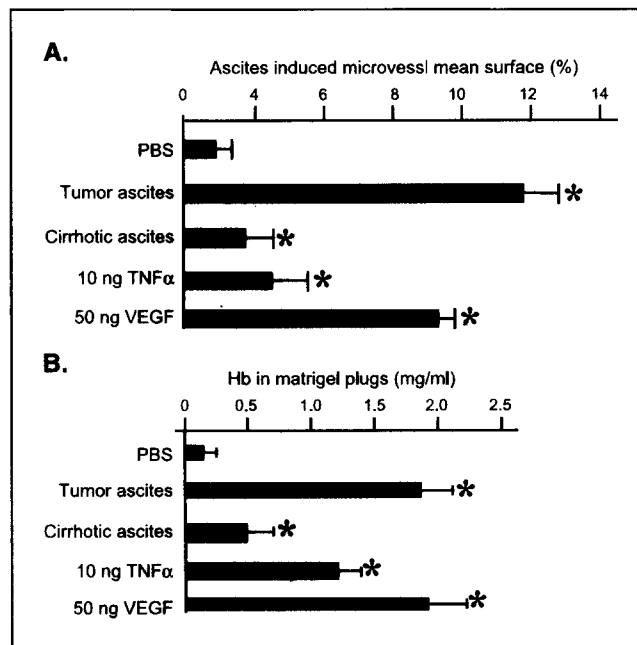
**ELISA.** Cytokines/chemokines in cell supernatants and ascites were detected with commercial kits (R&D Systems). Hemoglobin content in Matrigel plugs was detected with a commercial kit (Sigma, St. Louis, MO).

**Statistical Analysis.** Differences in cell surface molecule expression were determined by  $\chi^2$  test, and in other variables by unpaired *t* test, with *P* < 0.05 considered significant.

## Results

### Malignant Ascites Fluid Induces Potent *In vitro* Angiogenesis.

To determine the angiogenic factors in malignant ascites, we established an *in vivo* Matrigel assay model (16, 17). As expected, recombinant VEGF (*n* = 8) and tumor necrosis factor- $\alpha$  (*n* = 8) induced a significant angiogenesis (positive control; \*, *P* < 0.001, compared with PBS). Interestingly, both malignant ascites fluid (*n* = 12) and nontumor ascites (idiopathic cirrhosis; *n* = 6) induced *in vivo* angiogenesis (\*, *P* < 0.001, compared with PBS; Fig. 1). The percentage of microvessel surfaces (Fig. 1A) is correlated with the hemoglobin contents per Matrigel (Fig. 1B; refs. 14, 25). However, malignant ascites were thrice more powerful to induce angiogenesis *in vivo* than cirrhotic ascites (Fig. 1). These data showed that malignant ascites contained angiogenic factor(s).

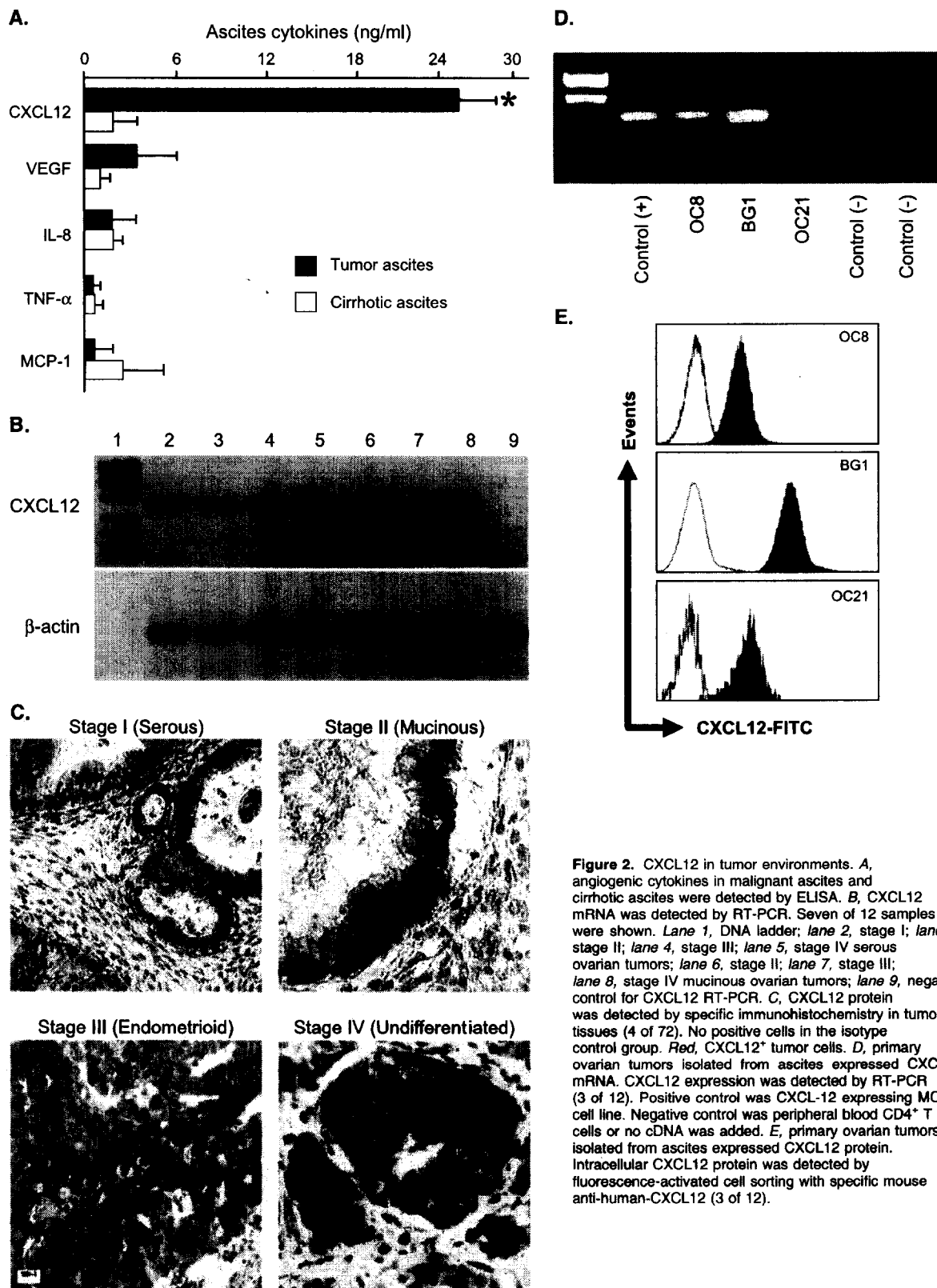


**Figure 1.** Malignant ascites fluid induces potent *in vivo* angiogenesis. C57 mice were inoculated with Matrigel plugs bearing malignant ascites fluid, cirrhotic ascites (0.5 mL), the indicated cytokines, or PBS. Day 12 Matrigel plugs were removed to study neovascularization as described in Methods. **A**, microvessel surface area in Matrigel plugs was quantified and expressed as % microvessel surface area as we described. **B**, Hb contents in Matrigel plugs were detected with a commercial kit. \*, *P* < 0.05, compared with PBS.

**CXCL12 in Malignant Ascites.** To determine the angiogenic factors in malignant ascites, we screened malignant ascites for the important identified angiogenic factors. We observed significant amounts of VEGF (3.1 ng/mL), IL-8 (1.2 ng/mL), and moderate amounts of tumor necrosis factor- $\alpha$  (0.23 ng/mL), and MCP-1 (0.27 ng/mL) in malignant ascites (*n* = 28; Fig. 2A). Strikingly, we detected high level of CXCL12 (25 ng/mL) in malignant ascites (*n* = 28; \*, *P* < 0.001, compared with other cytokines; Fig. 2A). Cirrhotic ascites (*n* = 6) contained moderate levels of these detected cytokines, suggesting that CXCL12 and VEGF may not be as critical as malignant ascites in inducing angiogenesis.

**CXCL12 Expression in Tumor Tissues and Primary Tumor Cells.** Consistent with our previous report (13), we observed that ovarian tumor tissues expressed potent CXCL12 mRNA (*n* = 12; Fig. 2B). Quantitative RT-PCR (20) revealed no significant difference of CXCL12 mRNA in tumors between FIGO stages I, II, III, and IV, as well as in tumors with different histology, including serous, mucinous, endometrioid, and undifferentiated ovarian tumors (Fig. 2B, data not shown). Immunohistochemistry analysis further confirmed that 100% ovarian tumor tissues expressed CXCL12 protein (*n* = 72; stage I, *n* = 14; stage II, *n* = 12; stage III, *n* = 28; stage IV, *n* = 18; Fig. 2C). No positive cells were observed in tissues stained with isotype antibody (data not shown). Notably, the level of CXCL12 protein expression was relatively variable and was not significantly different between different donors, between different disease stages, and between different tumor histologic types (Fig. 2C).

We further established 12 ovarian epithelial tumor cell lines from tumor ascites. We observed that all the primary tumor cell lines strongly expressed CXCL12 mRNA (*n* = 12; Fig. 2D). Intracellular



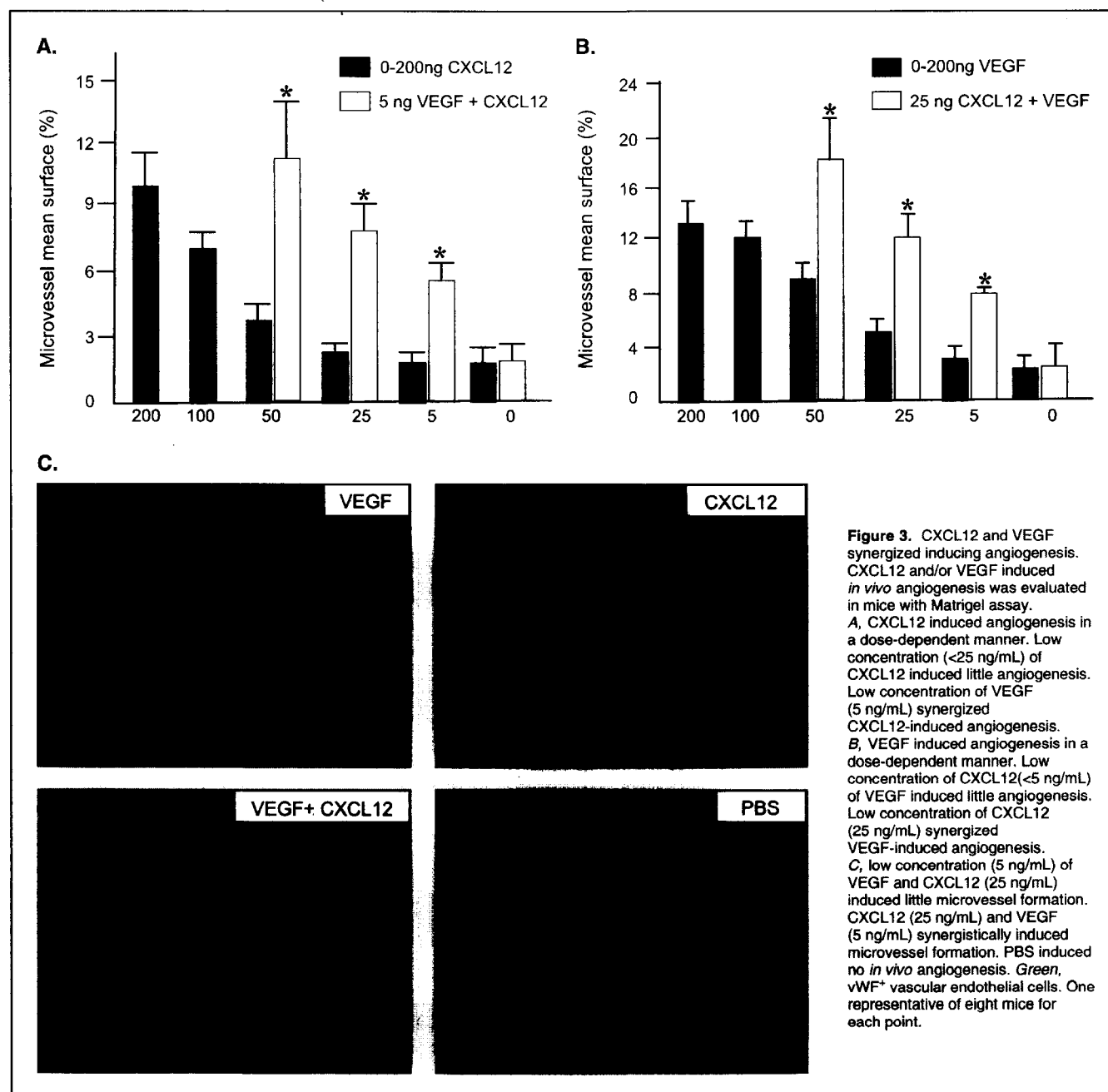
**Figure 2.** CXCL12 in tumor environments. **A**, angiogenic cytokines in malignant ascites and cirrhotic ascites were detected by ELISA. **B**, CXCL12 mRNA was detected by RT-PCR. Seven of 12 samples were shown. Lane 1, DNA ladder; lane 2, stage I; lane 3, stage II; lane 4, stage III; lane 5, stage IV serous ovarian tumors; lane 6, stage II; lane 7, stage III; lane 8, stage IV mucinous ovarian tumors; lane 9, negative control for CXCL12 RT-PCR. **C**, CXCL12 protein was detected by specific immunohistochemistry in tumor tissues (4 of 72). No positive cells in the isotype control group. Red, CXCL12<sup>+</sup> tumor cells. **D**, primary ovarian tumors isolated from ascites expressed CXCL12 mRNA. CXCL12 expression was detected by RT-PCR (3 of 12). Positive control was CXCL12 expressing MCF-7 cell line. Negative control was peripheral blood CD4<sup>+</sup> T cells or no cDNA was added. **E**, primary ovarian tumors isolated from ascites expressed CXCL12 protein. Intracellular CXCL12 protein was detected by fluorescence-activated cell sorting with specific mouse anti-human-CXCL12 (3 of 12).

staining showed that these primary tumor cell lines actively expressed intracellular CXCL12 ( $n = 12$ ; Fig. 2E). Therefore, ovarian tumor cells are the major cellular source in tumor environment.

**CXCL12 and VEGF Synergistically Induced *In vivo* Angiogenesis.** To determine the role of each individual cytokine in ascites-mediated *in vivo* angiogenesis (Fig. 1), we tested the *in vivo* angiogenesis in Matrigel assay with recombinant cytokines. CXCL12 induced a significant angiogenesis in a dose dependent manner ( $n = 7-10$  for each point; Fig. 3A). Notably, the effective angiogenic concentrations of CXCL12 were superior to 25 ng/mL (Fig. 3A). We detected 25 ng/mL CXCL12 in malignant ascites (Fig. 2A). The data indicates that high concentration of CXCL12 is angiogenic *in vivo*, whereas pathologic concentration of CXCL12 is not.

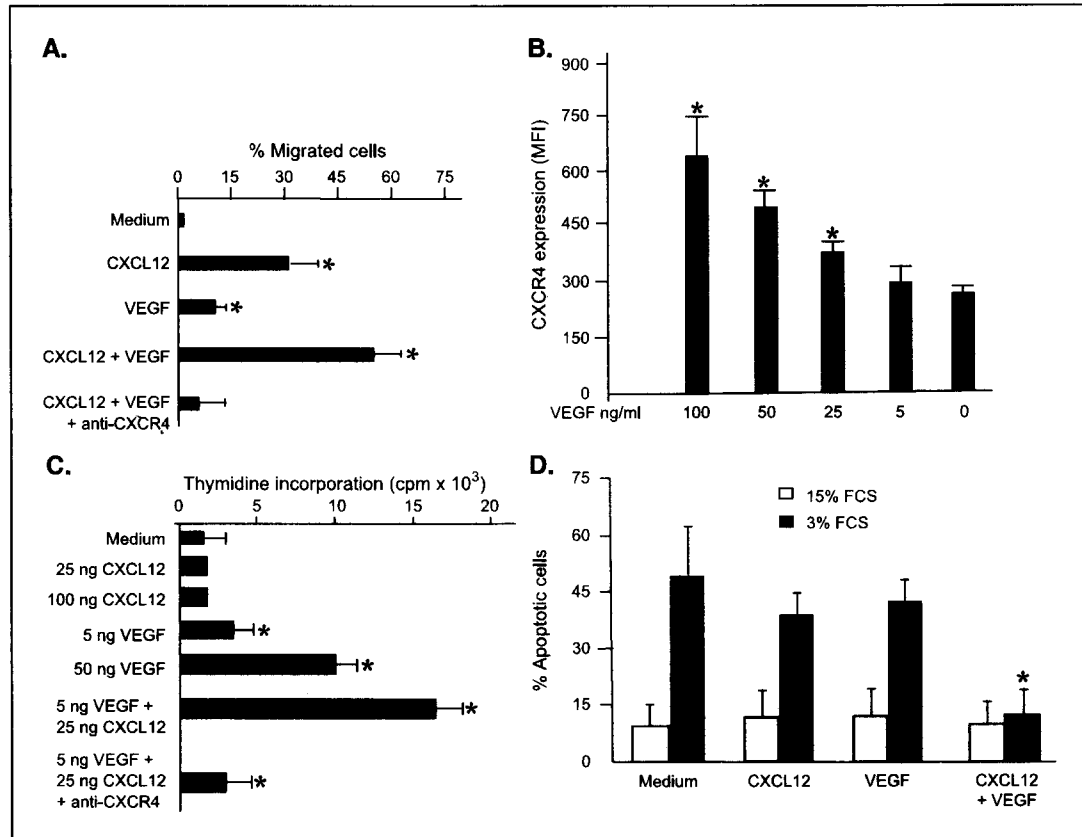
As expected, recombinant VEGF induced a significant angiogenesis in a dose dependent manner ( $n = 7-10$  for each point; Fig. 3B). However, consistent with other reports (14, 16), the effective angiogenic concentrations of VEGF were superior to 5 ng/mL in this experimental model (Fig. 3B). We detected 3.1 ng/mL VEGF in malignant ascites (Fig. 2A). The data indicates that pathologic concentration of VEGF is not able to induce a relevant *in vivo* angiogenesis in our model.

We hypothesized that tumor-derived VEGF and CXCL12 synergized to induce *in vivo* angiogenesis. We first examined whether VEGF synergized CXCL12-induced angiogenesis. Strikingly, 5 ng of VEGF significantly increased the angiogenic effects of CXCL12 (5-50 ng/mL) in a dose dependent



**Figure 3.** CXCL12 and VEGF synergized inducing angiogenesis. CXCL12 and/or VEGF induced *in vivo* angiogenesis was evaluated in mice with Matrigel assay. **A**, CXCL12 induced angiogenesis in a dose-dependent manner. Low concentration (<25 ng/mL) of CXCL12 induced little angiogenesis. Low concentration of VEGF (5 ng/mL) synergized CXCL12-induced angiogenesis. **B**, VEGF induced angiogenesis in a dose-dependent manner. Low concentration of CXCL12 (<5 ng/mL) of VEGF induced little angiogenesis. Low concentration of CXCL12 (25 ng/mL) synergized VEGF-induced angiogenesis. **C**, low concentration (5 ng/mL) of VEGF and CXCL12 (25 ng/mL) induced little microvessel formation. CXCL12 (25 ng/mL) and VEGF (5 ng/mL) synergistically induced microvessel formation. PBS induced no *in vivo* angiogenesis. Green, vWF+ vascular endothelial cells. One representative of eight mice for each point.

**Figure 4.** CXCL12 and VEGF synergistically promote angiogenic function of vascular endothelial cells. **A**, CXCL12 and VEGF synergistically induced HUVEC migration. CXCL12, 100 ng/mL; VEGF, 5 ng/mL. **B**, VEGF up-regulated CXCR4 expression on HUVEC. CXCR4 expression was analyzed by fluorescence-activated cell sorting. 100% cells express CXCR4. **MFI**, mean fluorescence intensity. One representative of six. **C**, CXCL12 synergized VEGF-mediated HUVEC proliferation. Cell proliferation was detected by thymidine incorporation (cpm). **D**, CXCL12 and VEGF synergistically protect HUVEC apoptosis. HUVEC were cultured with medium containing 3% and 15% FCS for 24 hours. CXCL12 (100 ng/mL) and VEGF (5 ng/mL) were added into the culture. Cellular apoptosis was analyzed by annexin V and 7-AAD staining and expressed as % apoptotic cells.



manner ( $n = 8$  for each point; \*,  $P < 0.001$ , compared with CXCL12 alone; Fig. 3A). The data indicate that pathologic concentration of VEGF synergized CXCL12-induced angiogenesis.

We next examined whether CXCL12 synergized VEGF-induced angiogenesis. Strikingly, 25 ng of CXCL12 significantly increased the angiogenic effects of VEGF (5–50 ng/mL) in a dose-dependent manner ( $n = 8$  for each group; \*,  $P < 0.001$ , compared with VEGF alone; Fig. 3B). The data indicate that pathologic concentration of CXCL12 synergized VEGF-induced angiogenesis. Histologic analysis showed significant vascular channel formation and tortuous neovessels in Matrigel plugs containing 25 ng of CXCL12 plus 5 ng of VEGF (Fig. 3C; ref. 17), but few microvessel formation in Matrigel plugs containing 25 ng of CXCL12 or 5 ng of VEGF. No microvessel formation was observed in Matrigel plugs containing PBS ( $n = 8$  for each group; Fig. 3C; ref. 25). Therefore, tumor-derived CXCL12 and VEGF likely form a synergistic angiogenic pathway *in vivo*.

**CXCL12 and VEGF Synergize to Promote Angiogenic Function of Vascular Endothelial Cells.** We next examined the synergistic mechanism by which CXCL12 and VEGF induced angiogenesis. Vascular endothelial cell migration is a critical step of tumor angiogenesis (4). We studied the directional migration of HUVEC. Both CXCL12 and VEGF induced a notable migration of HUVEC ( $n = 6$ ; \*,  $P < 0.0001$ , compared with medium for all; Fig. 4A). Interestingly, CXCL12-mediated migration was significantly more efficient in the presence of low concentration of VEGF (5 ng). Preincubation with a neutralizing antibody against CXCR4 completely disabled CXCL12-mediated HUVEC migration in the presence of VEGF, confirming the involvement of CXCR4. Further experiments showed that VEGF increased CXCR4 expression on

HUVEC ( $n = 5$ ; \*,  $P < 0.001$ , compared with medium; Fig. 4B), indicating that VEGF sensitized CXCL12-mediated migration of vascular endothelial cells through up-regulating CXCR4.

Vascular endothelial cell growth is important for tumor angiogenesis. We examined whether CXCL12 and VEGF could synergize to stimulate HUVEC proliferation. Unexpectedly, 25 to 100 ng of CXCL12 induced little HUVEC proliferation. VEGF induced HUVEC proliferation in a dose-dependent manner ( $n = 8$ ; \*,  $P < 0.001$ , compared with medium or CXCL12 alone). Interestingly, pathologic concentrations of VEGF (5 ng) and CXCL12 (25 ng) were significantly more efficient to induce vascular endothelial cell proliferation than VEGF alone ( $n = 5$ ; \*,  $P < 0.001$ , compared with 5 ng VEGF; Fig. 4C). Again, preincubation with anti-CXCR4 completely cancelled the VEGF-mediated proliferation, which was sensitized by CXCL12 (Fig. 4C). Thus, tumor-derived CXCL12 sensitizes VEGF-mediated vascular endothelial cell expansion.

Survival of vascular endothelial cells is critical for forming stable neovascularization. Deprivation of nutrients results in vascular endothelial cell apoptosis (14). We show that tumor environmental CXCL12 protects plasmacytoid dendritic cells from apoptosis (13). We hypothesize that CXCL12 and VEGF synergistically protect vascular endothelial cell apoptosis. To test this hypothesis, fresh HUVEC were cultured with different concentrations of FCS medium. FCS medium (3%) induced 50% apoptotic cells ( $n = 6$ ;  $P < 0.01$ , compared with 15% medium; Fig. 4D). CXCL12 and VEGF independently and marginally decreased the percentage of apoptotic cells induced by sera starvation ( $n = 6$ ;  $P < 0.05$ , compared with 15% FCS; Fig. 4D). Strikingly, CXCL12 plus VEGF efficiently reduced the percentage of apoptotic cells induced by 3% FCS medium

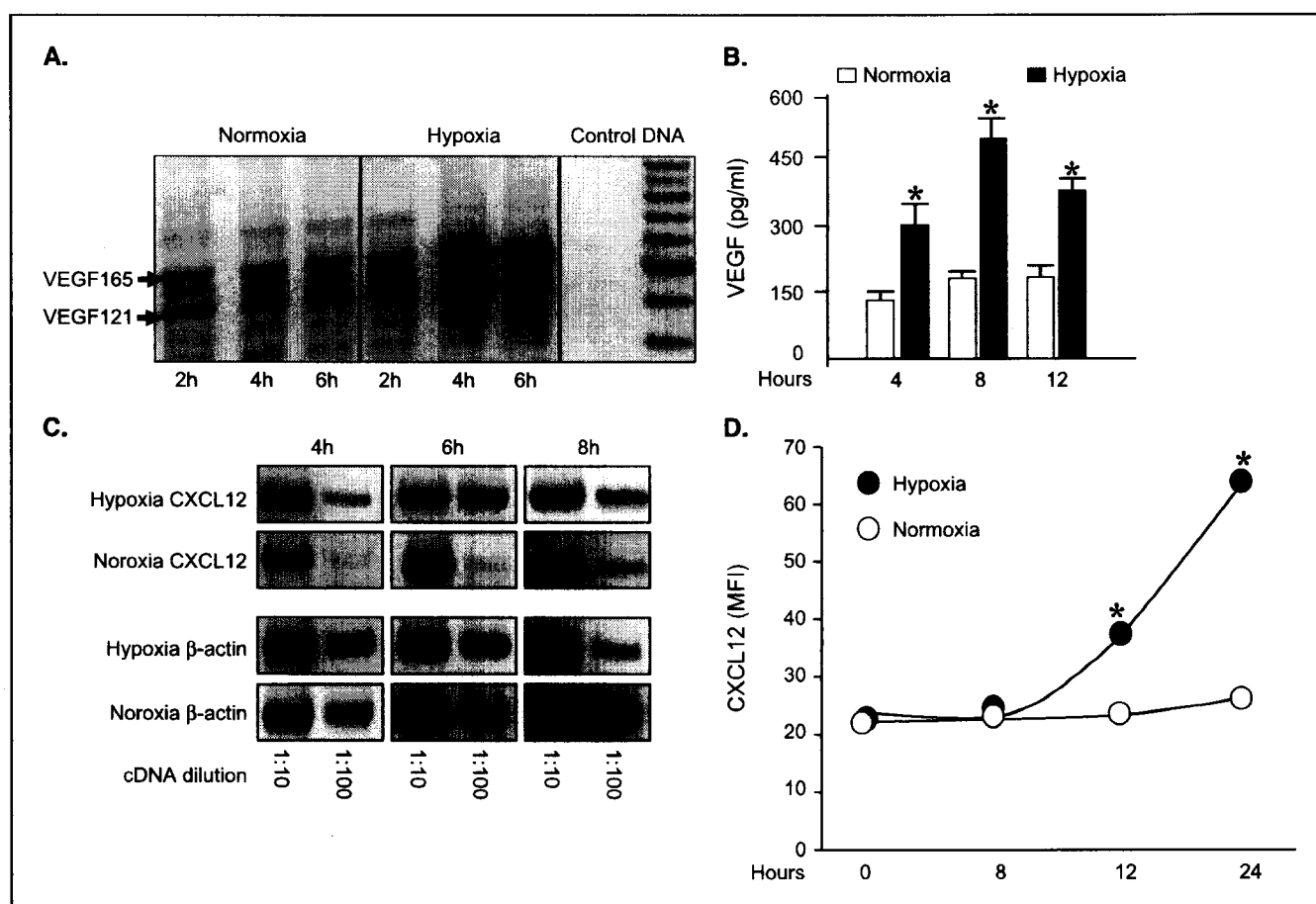
( $n = 6$ ;  $P < 0.001$ , compared with VEGF or CXCL12 alone; Fig. 4D), suggesting that VEGF and CXCL12 synergistically protected vascular endothelial cell apoptosis. The data indicate that multiple mechanisms are implicated in the *in vivo* synergistic angiogenic induction of CXCL12 and VEGF.

**Hypoxia Triggered CXCL12 and VEGF Production.** After determining that CXCL12 and VEGF form a synergistic angiogenic pathway, we next examined the potential regulatory mechanisms for this synergistic pathway. It is well documented that hypoxia induces VEGF production by tumor cells (4, 26, 27). We confirmed this finding. We showed that 4 to 6 hours after exposure to hypoxia, the level of VEGF165 and VEGF121 mRNA was 50- and 2.5-fold higher, respectively, in hypoxia-treated tumor cells than normoxia-treated tumor cells ( $n = 4$ ; Fig. 5A). Hypoxia particularly triggered the expression of VEGF121 and VEGF165, but not VEGF189 and VEGF206 (Fig. 5A). The hypoxia-induced VEGF was maintained for >24 hours (data not shown). As confirmation, hypoxia-treated ovarian tumor cells released more VEGF protein than normoxia ( $n = 8$ ,  $P < 0.001$ ; Fig. 5B). Furthermore, 4 hours after exposure to hypoxia, CXCL12 mRNA was significantly induced ( $n = 12$ ; Fig. 5C). At 6 hours, the level of CXCL12 mRNA was 100-fold higher in hypoxia than normoxia ( $n = 12$ ; Fig. 5C). We observed similar

results in one commercialized ovarian tumor cell line (BG-1) and three primary ovarian tumors cell lines (OC8, OC21, and OC38) established in the laboratory. As confirmation, intracellular staining showed that the level of CXCL12 protein was significantly higher in tumor cells exposed to hypoxia than normoxia ( $n = 8$ ,  $P < 0.001$ , compared with normoxia; Fig. 5D). Therefore, hypoxia activates the synergistic angiogenic pathway between VEGF and CXCL12 through synchronously triggering VEGF and CXCL12 production.

## Discussion

CXCL12 was originally isolated from murine bone marrow stromal cells (28), and described for its activity as a chemotactic cytokine for leukocytes (29, 30), CD34<sup>+</sup> progenitor cells (31–34), platelets (35, 36), and stem cells (33, 37). We previously showed that human ovarian epithelial tumor cells express high levels of CXCL12. Tumor-derived CXCL12 contributes to plasmacytoid dendritic cell trafficking and accumulation in tumor microenvironment (13). Tumor environmental plasmacytoid dendritic cells induce neoangiogenesis through IL-8 and tumor necrosis factor- $\alpha$  (25). We now show for the first time that pathologic concentration of



**Figure 5.** Hypoxia induces CXCL12 and VEGF production. Ovarian tumor cells (OC8) were exposed to normoxic (21% O<sub>2</sub>) or hypoxic (1% O<sub>2</sub>) for different times. **A**, hypoxia induced VEGF mRNA expression in tumor cells. Nested RT-PCR was done as described in Materials and Methods. VEGF165, 572 bp; VEGF121, 440 bp. **Control**, no DNA was added; **DNA**, DNA ladder. One of four representatives. **B**, hypoxia induced VEGF production by tumor cells. VEGF was detected in the culture supernatants by ELISA kit. \*,  $P < 0.001$ , compared with normoxia. **C**, hypoxia induced CXCL12 mRNA expression in tumor cells. RT-PCR was done as described in Materials and Methods. One of six representatives. **D**, hypoxia induced CXCL12 protein by tumor cells. CXCL12 was analyzed by fluorescence-activated cell sorting. **MFI**, mean fluorescence of intensity of CXCL12 expression.

tumor-derived CXCL12 plus VEGF synergistically induce potent neovascularization *in vivo*. The data suggest that this synergistic pathway would predominantly contribute to tumor vascularization *in vivo* in a real situation. The notion is supported by four lines of evidence: (1) High concentrations, but not pathologic concentrations of VEGF or CXCL12 induce angiogenesis in our *in vivo* model. (2) Pathologic concentrations of CXCL12 plus VEGF synergistically induce angiogenesis *in vivo*. (3) Hypoxia synchronously triggers both VEGF and CXCL12 production by tumor cells. (4) CXCL12 and VEGF synergistically promote vascular endothelial cell function, including migration, expansion, and survival. In support of the *in vivo* synergic effects of tumor-derived CXCL12 and VEGF in angiogenesis that we report here, the interaction between recombinant CXCL12 and VEGF has been described in previous *in vitro* studies, including *in vitro* cultured umbilical vein endothelial cells (38, 39), lymphohematopoietic cells (40), and breast cancer cell lines (41). However, direct evidence showing the *in vivo* synergistic angiogenesis between VEGF and CXCL12 is missing.

CXCL12 does not contain the  $\text{NH}_2$ -terminal Glu-Leu-Arg (ELR) motif with angiogenic function (42). The angiogenic potential of CXCL12 has been suggested in different settings of *in vitro* experiments (39, 43–46). Our *in vivo* data indicate that high concentration of CXCL12 directly induce angiogenesis. In support, mice lacking CXCL12 or CXCR4 have defective vascular system development (47, 48). S.c. injection of high concentration recombinant CXCL12 induced formation of local small blood vessels (38). However, our *in vitro* and *in vivo* data indicate that pathologic concentrations of CXCL12 and VEGF are not able to induce a pronounced *in vivo* angiogenesis, whereas pathologic concentrations of CXCL12 and VEGF induce potent *in vivo* angiogenesis in a synergistic manner. Apart from tumors (13), CXCL12 is constitutively expressed in stromal cells, vascular endothelial cells, osteoclast, and some epithelial cells, suggesting that CXCL12 would be important in different physiologic angiogenesis settings.

Anti-CXCR4 treatment significantly decreases the progression and metastasis of cancers in mice (49). The current explanation is that anti-CXCR4 blocks CXCR4 expressing tumor migration to CXCL12 expressing tissues (49–52). Our current data indicate that CXCL12/CXCR4 system is significantly involved in tumor angiogenesis by synergizing with VEGF. Thus, additional explanation is that anti-CXCR4 blocks tumor angiogenesis mediated by CXCL12 and VEGF synergistic pathway and in turn reduces tumor metastasis. In

further support of this notion, VEGF has been reported to be an autocrine survival factor and protects breast cancer cells from apoptosis induced by serum deprivation (53, 54). Hypoxia induces tumor VEGF (53). We now show for the first time that hypoxia triggers tumor CXCL12 expression. It may be a common mechanism in many human tumors that hypoxia synchronously induces VEGF and CXCL12, and VEGF and CXCL12 in turn synergistically protect tumor cell or vascular endothelial cell apoptosis from hypoxia in tumor environment and synergistically promote tumor vascularization and growth. Ovarian tumor cells produce a large amount of CXCL12 and VEGF, which are released into peritoneal cavity. The synergistic pathway between CXCL12 and VEGF would explain, at least partially the extensive tumor vascularization and metastasis in peritoneal cavity in most advanced ovarian cancers.

Therapeutically, the synergic axis between CXCL12 and VEGF has been ignored in prior antitumor angiogenesis strategies. Importantly, early human clinical cancer treatment trials with antiangiogenic molecules have not shown significant benefits predicted from preclinical models (3, 7, 8). More strikingly, recent reports (55–57) suggest that certain angiogenesis inhibitors (or antagonists) alone, by depriving tumors of oxygen, could have an unintended effect: promotion of tumor metastasis by increasing CXCR4 expression. These results reflect our growing understanding of the complexity of the tumor angiogenic process and suggest that blocking both CXCR4 and VEGF will be a novel, efficient strategy to treat human cancers.

In summary, we show in this report that tumors produced functional CXCL12 and VEGF, and tumor-derived CXCL12 and VEGF formed a synergistic angiogenesis axis *in vivo*, and hypoxia activates this axis through synchronously triggering tumor CXCL12 and VEGF production. The study suggests that CXCL12 and VEGF formed synergistic angiogenic pathway is critical for tumor neovascularization, and targeting both CXCL12 and VEGF signals may be a novel, efficient strategy for treating human cancers.

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# Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival

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Regulatory T ( $T_{reg}$ ) cells mediate homeostatic peripheral tolerance by suppressing autoreactive T cells. Failure of host antitumor immunity may be caused by exaggerated suppression of tumor-associated antigen-reactive lymphocytes mediated by  $T_{reg}$  cells; however, definitive evidence that  $T_{reg}$  cells have an immunopathological role in human cancer is lacking. Here we show, in detailed studies of  $CD4^+CD25^+FOXP3^+$   $T_{reg}$  cells in 104 individuals affected with ovarian carcinoma, that human tumor  $T_{reg}$  cells suppress tumor-specific T cell immunity and contribute to growth of human tumors *in vivo*. We also show that tumor  $T_{reg}$  cells are associated with a high death hazard and reduced survival. Human  $T_{reg}$  cells preferentially move to and accumulate in tumors and ascites, but rarely enter draining lymph nodes in later cancer stages. Tumor cells and microenvironmental macrophages produce the chemokine CCL22, which mediates trafficking of  $T_{reg}$  cells to the tumor. This specific recruitment of  $T_{reg}$  cells represents a mechanism by which tumors may foster immune privilege. Thus, blocking  $T_{reg}$  cell migration or function may help to defeat human cancer.

T cells targeted at tumor-associated antigens (TAAs) are readily detectable in blood, tumors and draining lymph nodes of individuals with cancer, even at late stages of disease. These tumor-specific T cells<sup>1–4</sup> can be used to establish functional TAA-specific T cell lines, which kill autologous tumor cells *in vitro* and *in vivo*<sup>5</sup>. The spontaneous clearance of established tumors by endogenous immune mechanisms is, however, rare. In addition, vaccine-induced increases in TAA-specific T cells do not always coincide with tumor regression<sup>1,6–8</sup>. Thus, it is clear that established tumors induce immune tolerance to escape destruction<sup>9–13</sup>, although the underlying mechanisms are not well defined.

Indirect evidence suggests that  $CD4^+CD25^+$  T cells ( $T_{regs}$ ) are important in suppressing TAA-specific immunity<sup>14–21</sup>.  $CD4^+CD25^+$  T cells are present in malignant effusions<sup>22,23</sup> and blood<sup>24</sup> of individuals with various types of cancer, and they suppress nonspecific T cell responses *in vitro*. However, direct evidence supporting a role for  $T_{reg}$  cells in the immunopathogenesis of human cancers is lacking, as is specific knowledge of the trafficking mechanisms for  $T_{reg}$  cells.

## RESULTS

### $CD4^+CD25^+$ T cells in malignant ascites

In individuals with untreated malignant ovarian epithelial cancers ( $n = 45$ ), we identified a substantial population of  $CD4^+CD25^+CD3^+$

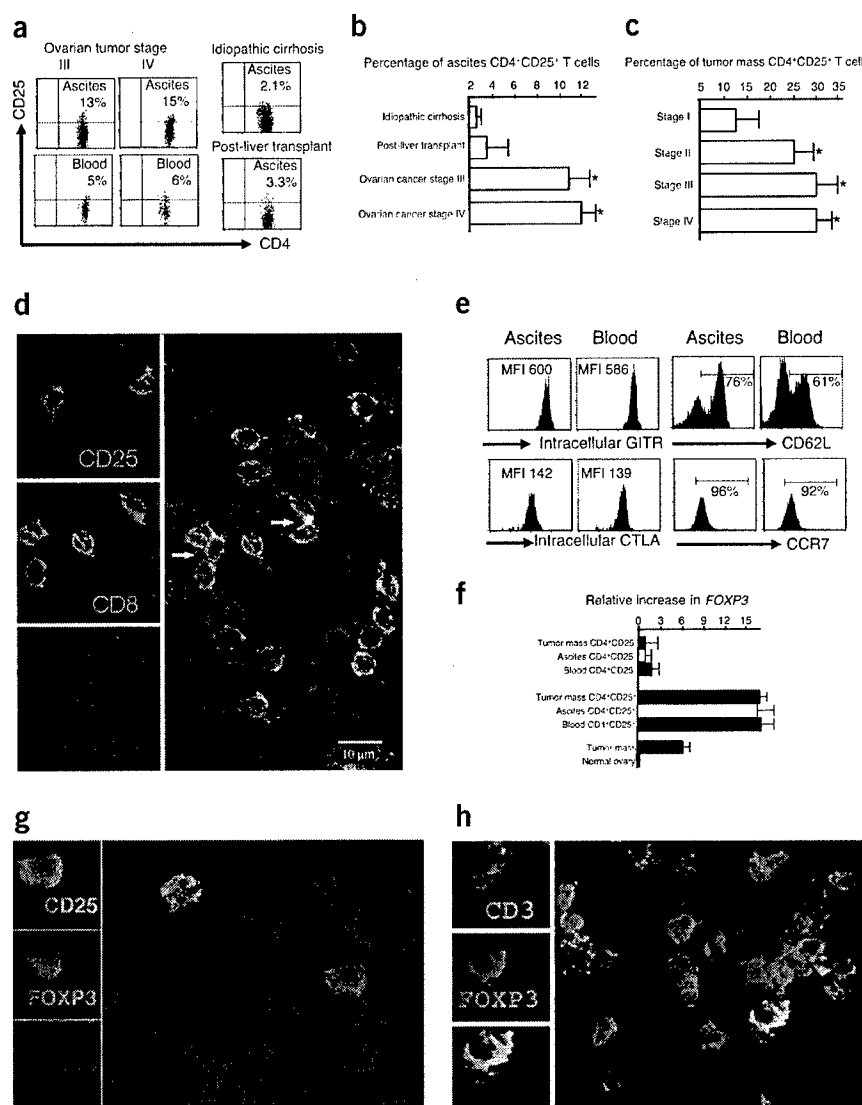
T cells (10–17% of all T cells) by flow cytometry (FACS analysis) in malignant ascites (Fig. 1a,b).  $CD4^+CD25^+CD3^+$  T cells were more abundant in ascites of individuals with stage III ( $n = 30$ ,  $P < 0.01$ ) and stage IV ( $n = 15$ ,  $P < 0.01$ ) tumors than in their blood (Fig. 1a,b). We observed a small fraction of  $CD4^+CD25^+CD3^+$  T cells (0.7–5.0%) in nonmalignant ascites from individuals with idiopathic cirrhosis ( $n = 6$ ) or who had undergone a liver transplant ( $n = 4$ ; Fig. 1a,b). There were fewer  $CD4^+CD25^+$  T cells in nonmalignant ascites ( $P < 0.01$ ) than in malignant ascites, however, supporting the idea that  $CD4^+CD25^+$  T cells accumulate in a tumor-specific manner in malignant ascites. Malignant ascites in stage I and II ovarian tumors is uncommon and was not available for study.

### $CD4^+CD25^+$ T cells in the solid tumor mass

To confirm our results in malignant ascites, we studied 104 tumor tissues from individuals with untreated ovarian epithelial cancers, including the 45 individuals described above. Consistent with results from malignant ascites, we identified a substantial accumulation of  $CD4^+CD25^+CD3^+$  T cells by multicolor confocal microscopic analysis in the tumor mass. Tumor-infiltrating  $CD4^+CD25^+$  T cells represented  $23 \pm 11\%$  (mean  $\pm$  s.e.m.) of tumor-infiltrating  $CD4^+$  T cells (Fig. 1c). The percentage of  $CD4^+CD25^+CD3^+$  T cells in  $CD4^+CD3^+$  T cells was

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**Figure 1** CD4<sup>+</sup>CD25<sup>+</sup> T cells in distinct tumor microenvironments. **(a,b)** CD4<sup>+</sup>CD25<sup>+</sup>CD3<sup>+</sup> cells preferentially accumulate in malignant ascites. Cells were analyzed by FACS with multicolor staining and gating on CD3<sup>+</sup>CD4<sup>+</sup> cells. **(a)** Two representative samples of malignant ascites from individuals with ovarian cancers ( $n = 45$  analyzed), and two samples of non-tumor ascites from control individuals ( $n = 10$ ). \* $P < 0.01$  versus nontumor ascites or blood. **(b)** Percentage of CD4<sup>+</sup>CD25<sup>+</sup> T cells in CD4<sup>+</sup>CD3<sup>+</sup> cells (mean  $\pm$  s.e.m.). \* $P < 0.001$  versus nontumor ascites. **(c,d)** CD4<sup>+</sup>CD25<sup>+</sup>CD3<sup>+</sup> cells accumulate in tumor mass. **(c)** Percentage of tumor mass CD4<sup>+</sup>CD25<sup>+</sup>CD3<sup>+</sup> cells quantified by confocal microscopic analysis. Results are expressed as in **b**. \* $P < 0.001$  versus stage I. **(d)** CD4<sup>+</sup>CD25<sup>+</sup>CD3<sup>+</sup> cells are in close contact with CD8<sup>+</sup>CD3<sup>+</sup> T cells in tumor tissues (arrows, stage III) *in vivo*. CD4<sup>+</sup>CD25<sup>+</sup>CD3<sup>+</sup> T cells were identified as CD3<sup>+</sup>CD8<sup>+</sup>CD25<sup>+</sup> T cells. White color indicates intimate association of CD4<sup>+</sup>CD25<sup>+</sup> T cells and CD8<sup>+</sup> T cells. A representative sample is shown ( $n = 53$ ). **(e-h)** Phenotype of tumor-associated CD4<sup>+</sup>CD25<sup>+</sup> T cells. **(e)** All (100%) of tumor ascites and blood CD4<sup>+</sup>CD25<sup>+</sup> T cells express intracellular GITR and CTLA-4, and most strongly express the lymphoid homing molecules CD62L and CCR7. Cells were gated on CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> cells ( $n = 5$  for each). MFI, mean fluorescence intensity. **(f)** Expression of *FOXP3* by human CD4<sup>+</sup>CD25<sup>+</sup> T cells. CD4<sup>+</sup>CD25<sup>+</sup> T cells and CD4<sup>+</sup>CD25<sup>-</sup> T cells were sorted by FACS from peripheral blood and from infiltrating T cells in the tumor mass and ascites. Real-time PCR for *FOXP3* was done in triplicate and relative fold changes were normalized to *GAPDH*. \* $P < 0.001$ , CD4<sup>+</sup>CD25<sup>+</sup> T cells versus CD4<sup>+</sup>CD25<sup>-</sup> T cells or normal ovary tissues. **(g,h)** FOXP3<sup>+</sup> T<sub>reg</sub> cells in ovarian tumor mass. Tissues were stained with antibodies to human FOXP3, human CD25 and CD8 **(g)**, or with antibodies to human FOXP3 and human CD3 **(h)** ( $n = 5$  for each). FOXP3<sup>+</sup>CD3<sup>+</sup> (T<sub>reg</sub>) cells are in close contact with FOXP3<sup>+</sup>CD8<sup>+</sup> T cells (mostly CD8<sup>+</sup> T cells). Scale bars, 40  $\mu$ m.

higher in later disease stages (stage II,  $n = 11$ ; stage III,  $n = 53$ ; stage IV,  $n = 33$ ) than in early disease stages (stage I,  $n = 7$ ;  $P < 0.001$  for all). In addition,  $75 \pm 17\%$  of CD4<sup>+</sup>CD25<sup>+</sup>CD3<sup>+</sup> T cells in the tumor mass were in proximity to infiltrating CD8<sup>+</sup> T cells ( $n = 32$ ; Fig. 1d), suggesting that physical contact between CD4<sup>+</sup>CD25<sup>+</sup> T cells and CD8<sup>+</sup> cytotoxic T cells mediates regulatory functions as proposed<sup>14–17</sup>.

CD4<sup>+</sup>CD25<sup>+</sup> T cells were undetectable in normal ovarian tissues from five control subjects without cancer (data not shown). Thus, these data show the accumulation of CD4<sup>+</sup>CD25<sup>+</sup> T cells in malignant ascites and tumor tissue in individuals with ovarian cancers.

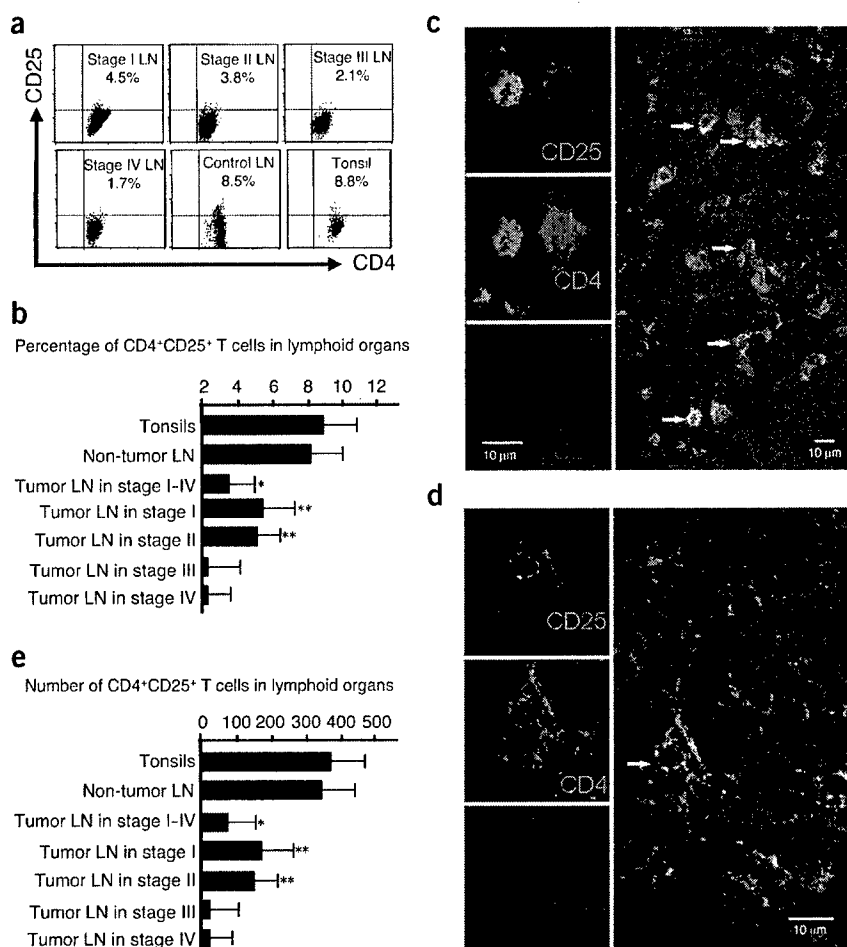
#### Phenotype of tumor-associated CD4<sup>+</sup>CD25<sup>+</sup> T cells

Freshly isolated CD4<sup>+</sup>CD25<sup>+</sup> T cells from tumor ascites and peripheral blood expressed similar levels of membrane glucocorticoid-induced tumor-necrosis factor (TNF) receptor family-related gene (GITR) and cytolytic T lymphocyte-associated antigen 4 protein (CTLA-4; data not shown). Because of their different localizations, we reasoned that tumor ascites and blood CD4<sup>+</sup>CD25<sup>+</sup> T cells expressed different levels of intracellular GITR and CTLA-4. Tumor ascites CD4<sup>+</sup>CD25<sup>+</sup> T cells expressed intracellular GITR and CTLA-4

(Fig. 1e), similar to circulating, CD4<sup>+</sup>CD25<sup>+</sup> T cells<sup>14–18</sup>. The majority of CD4<sup>+</sup>CD25<sup>+</sup> T cells in blood and malignant ascites expressed CD62L (>60%) and CCR7 (>90%; Fig. 1e).

*Foxp3* is crucial for the differentiation and function of mouse CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells<sup>25–27</sup>. Real-time PCR showed strong and equivalent expression of *FOXP3* mRNA in CD4<sup>+</sup>CD25<sup>+</sup> T cells isolated from malignant ascites ( $n = 7$ ), the tumor mass ( $n = 4$ ) and blood ( $n = 6$ ) from individuals with ovarian cancers, as compared with their CD4<sup>+</sup>CD25<sup>-</sup> T cells ( $P < 0.001$  for each comparison; Fig. 1f). We also observed strong *FOXP3* expression in ovarian tumor tissues ( $n = 10$ ,  $P < 0.001$ ), but not in normal ovarian tissues from controls without cancer ( $n = 5$ ; Fig. 1f). In support of this, we observed a substantial infiltration of CD8<sup>+</sup>FOXP3<sup>+</sup>CD25<sup>+</sup> cells into the tumor mass. All (100%) of the tumor-infiltrating FOXP3<sup>+</sup> cells were CD25<sup>+</sup> T cells, whereas  $90 \pm 8\%$  CD25<sup>+</sup> T cells were FOXP3<sup>+</sup> cells in the tumor mass ( $n = 5$ ; Fig. 1g). Consistent with our above observation (Fig. 1d), many of the FOXP3<sup>+</sup>CD25<sup>+</sup> cells ( $80 \pm 17\%$ ) were in close contact with CD8<sup>+</sup> T cells (Fig. 1g).

We also showed that 100% of the tumor-infiltrating FOXP3<sup>+</sup> cells were CD3<sup>+</sup> T cells ( $n = 5$ ; Fig. 1h). Therefore, tumor-infiltrating CD4<sup>+</sup>CD25<sup>+</sup>CD3<sup>+</sup> T cells express *FOXP3* and are often in close contact



**Figure 2** CD4<sup>+</sup>CD25<sup>+</sup> T cells in lymph nodes. (a) Representative FACS analysis of samples from tumor-draining lymph nodes (LN) from 36 affected individuals. (b) Percentage of CD4<sup>+</sup>CD25<sup>+</sup> T cells in CD4<sup>+</sup>CD3<sup>+</sup> cells (mean  $\pm$  s.e.m.). Lymphoid organ cells were analyzed by FACS with multicolor staining and gating on CD3<sup>+</sup>CD4<sup>+</sup> cells. \**P* < 0.01 versus nontumor lymph nodes (*n* = 6) or tonsils (*n* = 8). \*\**P* < 0.001 versus stage III or stage IV. (c, d) Large numbers of CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> cells are present in control lymphoid organs (c; *n* = 8), but few are present in tumor-draining lymph nodes (d; *n* = 8). Arrow and white color indicate triple-positive (CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>) cells. (e) Number of CD4<sup>+</sup>CD25<sup>+</sup> T cells in draining lymph nodes of tumors at various stages of disease. CD4<sup>+</sup>CD25<sup>+</sup>CD3<sup>+</sup> T cells were quantified by confocal microscopy. \**P* < 0.001 versus nontumor lymph nodes or tonsils. \*\**P* < 0.001 versus stage III or stage IV. Data show the number of CD4<sup>+</sup>CD25<sup>+</sup>CD3<sup>+</sup> cells per ten HPF. Scale bars, 10  $\mu$ m.

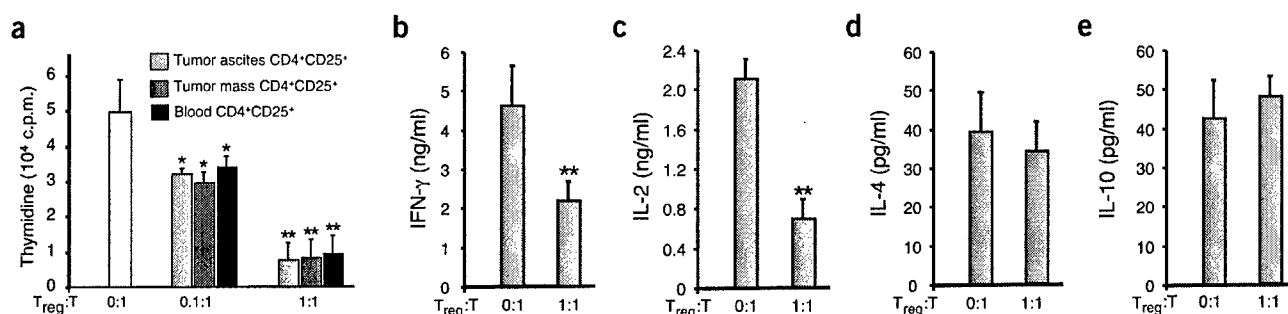
with CD8<sup>+</sup> T cells. Taken together, the CD4<sup>+</sup>CD25<sup>+</sup>CD3<sup>+</sup>GITR<sup>+</sup>CTLA-4<sup>+</sup>CCR7<sup>+</sup>FOXP3<sup>hi</sup> phenotype of tumor-associated CD4<sup>+</sup>CD25<sup>+</sup> T cells suggests that they have characteristics of T<sub>reg</sub> cells<sup>14,25–27</sup>.

#### CD4<sup>+</sup>CD25<sup>+</sup> T cells in tumor-draining lymph nodes

CD62L and CCR7 direct migrating cells to lymph nodes, where homeostatic T<sub>reg</sub> cells readily accumulate<sup>14,16,28</sup>. We therefore stud-

ied the content of T<sub>reg</sub> cells in tumor-draining lymph nodes from individuals with ovarian cancer (*n* = 36). We first compared the accumulation of CD4<sup>+</sup>CD25<sup>+</sup> T cells in tumor-draining lymph nodes to that in lymph nodes from individuals without cancer. Less than 5% of CD4<sup>+</sup> T cells in tumor-draining lymph nodes were CD4<sup>+</sup>CD25<sup>+</sup>, as assessed by FACS. This accumulation of CD4<sup>+</sup>CD25<sup>+</sup> T cells in tumor-draining lymph nodes was lower than in control lymph nodes (*n* = 6, *P* < 0.01) and tonsils (*n* = 8, *P* < 0.01; Fig. 2a,b). We were unable to obtain lymph nodes that did not drain

the tumor in individuals with ovarian cancer. We also examined accumulation of CD4<sup>+</sup>CD25<sup>+</sup> T cells in tumor-draining lymph nodes according to tumor stage. CD4<sup>+</sup>CD25<sup>+</sup> T cells in tumor-draining lymph nodes were less frequent in stage III (*n* = 14) or IV (*n* = 11) than in stage II (*n* = 5) or I (*n* = 6; *P* < 0.01 for III plus IV versus I plus II; Fig. 2a,b). We further quantified the absolute amount of CD4<sup>+</sup>CD25<sup>+</sup>CD3<sup>+</sup> cells in tumor-draining



**Figure 3** Tumor T<sub>reg</sub> cells suppress T cell activation *in vitro*. Tumor ascites CD3<sup>+</sup>CD25<sup>+</sup> T cells were stimulated with soluble antibody to CD3 plus monoclonal antibody to CD28 for 6 d. CD4<sup>+</sup>CD25<sup>+</sup> T cells from tumor ascites, the tumor mass or blood were added to the culture at the indicated ratio of regulatory (T<sub>reg</sub>) to responder (T) cells. (a) Tumor ascites and tumor mass CD4<sup>+</sup>CD25<sup>+</sup> T cells are as efficient as blood CD4<sup>+</sup>CD25<sup>+</sup> T cells at inhibiting T cell proliferation. White bar, no T<sub>reg</sub>. (b–e) Tumor ascites CD4<sup>+</sup>CD25<sup>+</sup> T cells inhibit T cell production of IFN- $\gamma$  (b) and IL-2 (c), but have no effect on IL-4 (d) or IL-10 (e) production (*n* = 5, \**P* < 0.05, \*\**P* < 0.01).

lymph nodes by multicolor confocal microscopic analysis. Consistent with FACS data, we observed numerous CD4<sup>+</sup>CD25<sup>+</sup>CD3<sup>+</sup> cells in control lymph nodes ( $n = 7$ ; Fig. 2c,e), but significantly fewer in tumor-draining lymph nodes ( $n = 36$ ;  $P < 0.001$ ; Fig. 2d,e), suggestive of limited movement of CD4<sup>+</sup>CD25<sup>+</sup>CD3<sup>+</sup> cells into tumor-draining lymph nodes, which further declined in later tumor stages. By contrast, the frequency of CD4<sup>+</sup>CD25<sup>+</sup> T cells increased in ascites and the solid tumor mass in later tumor stages (Fig. 1).

These data suggest that, in contrast to homeostatic CD4<sup>+</sup>CD25<sup>+</sup> T cells, which preferentially home to lymphoid tissue, CD4<sup>+</sup>CD25<sup>+</sup> T cells in individuals with cancer are preferentially recruited to the tumor mass and associated ascites, but not to locally draining lymph nodes.

### Tumor CD4<sup>+</sup>CD25<sup>+</sup> T cells suppress T cell activation

To determine whether the CD4<sup>+</sup>CD25<sup>+</sup> T cells in tumor ascites and tumor tissue were functional T<sub>reg</sub> cells, we used an *in vitro* cellular culture system<sup>23,29</sup>. When activated with soluble monoclonal antibody to CD3 plus monoclonal antibody to CD28, tumor ascites CD3<sup>+</sup>CD25<sup>+</sup> T cells responded with robust proliferation (Fig. 3a). Tumor CD4<sup>+</sup>CD25<sup>+</sup> T cells from malignant ascites and the tumor mass significantly inhibited this T cell proliferation in a dose-dependent manner ( $n = 5$ ,  $P < 0.05$ ; Fig. 3a).

We further observed that tumor ascites CD4<sup>+</sup>CD25<sup>+</sup> T cells suppressed the production of interferon- $\gamma$  (IFN- $\gamma$ ; Fig. 3b) and interleukin-2 (IL-2; Fig. 3c) by T cells. We observed little production of IL-4 (Fig. 3d) or IL-10 (Fig. 3e) by T cells in culture, and tumor ascites CD4<sup>+</sup>CD25<sup>+</sup> T cells had no significant effect on either (Fig. 3d,e). Tumor-associated CD4<sup>+</sup>CD25<sup>+</sup> T cells (from the tumor mass or ascites) and peripheral circulating CD4<sup>+</sup>CD25<sup>+</sup> T cells comparably affected T cell suppression (Fig. 3a). Tumor-associated and circulating CD4<sup>+</sup>CD25<sup>+</sup> T cells themselves proliferated poorly after activation with monoclonal antibody to CD3 plus monoclonal antibody to CD28 (<2,000 counts per min). Thus, tumor-associated CD4<sup>+</sup>CD25<sup>+</sup> T cells (from malignant ascites or the tumor mass) are functional T<sub>reg</sub> cells. Hereafter we refer to them as 'tumor T<sub>reg</sub> cells.'

### CCL22-mediated trafficking of T<sub>reg</sub> cells *in vitro*

We next studied mechanisms by which T<sub>reg</sub> cells might migrate into tumors. Malignant ascites induced significant migration (Fig. 4a) and transmigration (Fig. 4b) of T<sub>reg</sub> cells *in vitro* ( $P < 0.01$  for each). Ovarian cancers produce CXCL12, which mediates the trafficking of plasmacytoid dendritic cells to tumors<sup>30</sup>; however, a neutralizing monoclonal antibody to human CXCR4, which blocks CXCL12-mediated chemotaxis<sup>30</sup>, did not block ascites-induced T<sub>reg</sub> cell migration (Fig. 4a). A saturated concentration of monoclonal antibody to -human CCL22, but not monoclonal antibody to CCL17, significantly blocked ascites-induced T<sub>reg</sub> cell migration and transmigration (Fig. 4a,b). In support of this, recombinant CCL22 induced significant T<sub>reg</sub> cell migration ( $P < 0.01$  versus control), which was efficiently blocked by a saturated concentration of antibody to CCL22 (Fig. 4a,b).

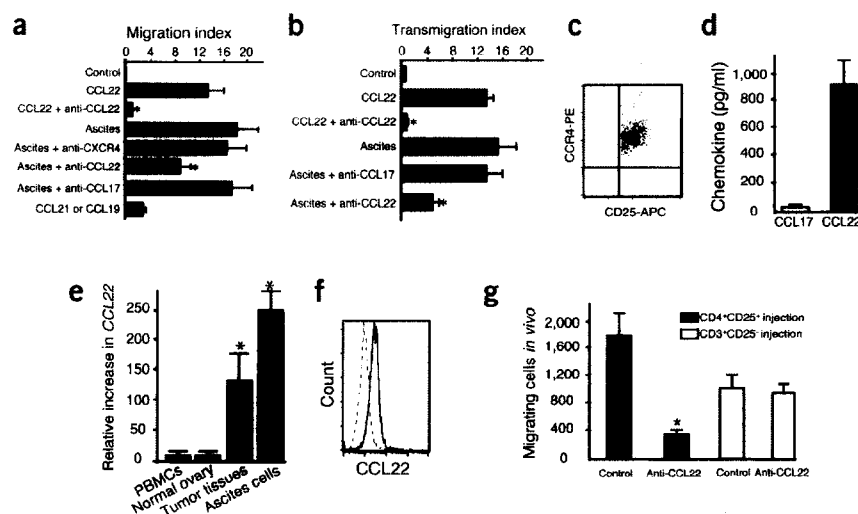
All tumor T<sub>reg</sub> cells expressed CCR4 (mean fluorescence intensity  $116 \pm 21$ ,  $n = 8$ ; Fig. 4c), a receptor for CCL22 and CCL17. Although tumor-associated T<sub>reg</sub> cells expressed CCR7 (Fig. 1f), recombinant CCL19 and CCL21, which are ligands of CCR7, were significantly less efficient than CCL22 in mediating the migration of tumor-associated T<sub>reg</sub> cells ( $P < 0.001$  versus CCL22 or ascites; Fig. 4a). Thus, CCL22 mediates T<sub>reg</sub> cells trafficking *in vitro* and may recruit T<sub>reg</sub> cells to the tumor.

### Tumor cells and environmental macrophages produce CCL22

Consistent with the *in vitro* migration data (Fig. 4a,b), we detected large amounts of CCL22 but not CCL17 in tumor ascites ( $n = 18$ ) by specific enzyme-linked immunosorbent assay (ELISA; Fig. 4d). Tumor tissue ( $n = 16$ ) and ascites cells ( $n = 8$ ), but not peripheral blood mononuclear cells (PBMCs;  $n = 8$ ) or normal ovaries ( $n = 5$ ), strongly expressed CCL22 mRNA ( $P < 0.001$  for all versus PBMCs or normal ovaries; Fig. 4e). Primary tumor ascites macrophages ( $n = 8$ ) were uniformly positive for intracellular CCL22 (Fig. 4f) and secreted CCL22 into culture supernatants ( $275 \pm 121$  pg/ml,  $n = 6$ ). Thus, ovarian tumors and microenvironmental macrophages are major sources of CCL22 and may induce T<sub>reg</sub> cells trafficking to tumors.

**Figure 4** A CCL22-CCR4 signal mediates CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells migration. (a) CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells migrate in response to malignant ascites or recombinant CCL22. A specific antibody to CCL22 significantly inhibits CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cell migration. Results are the mean  $\pm$  s.d. (b) Similar results are observed for CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cell adhesion or transmigration ( $n = 4$  independent experiments; \* $P < 0.001$  versus control in a and b). (c) Tumor CD4<sup>+</sup>CD25<sup>+</sup> T cells express high levels of CCR4. FACS analysis was gated on CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> malignant ascites cells ( $n = 8$ ). (d) CCL22 is detected in malignant ascites by ELISA ( $n = 18$ ). (e) Real-time RT-PCR shows significant CCL22 expression in tumor tissues and tumor ascites cells (\* $P < 0.001$  versus PBMCs or normal ovary). (f) Primary tumor ascites macrophages express intracellular CCL22 protein. Dotted line, isotype staining; solid line, CCL22 staining (one representative analysis is shown;  $n = 8$ ). (g) CCL22-CCR4 signals mediate trafficking of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells to the tumor *in vivo*.

Human T cells and monoclonal antibody to CCL22 were injected into mice and tumors were extracted after 48 h to detect human T cell trafficking by FACS. Monoclonal antibody to CCL22 significantly inhibits trafficking of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells but not CD3<sup>+</sup>CD25<sup>+</sup> T cells to tumors ( $n = 7-10$  per group, \* $P < 0.01$  versus control). Data show the total recovery of T<sub>reg</sub> cells in  $10^8$  cells analyzed.



**CCL22 induces migration of T<sub>reg</sub> cells into tumor *in vivo***

We reconstituted human nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice with primary human ovarian tumors as described<sup>5</sup>, and adoptively transferred human T<sub>reg</sub> cells into these tumor-bearing mice. We observed numerous xenotransplanted human T<sub>reg</sub> cells migrating into tumor tissues (Fig. 4g). *In vivo* treatment with monoclonal antibody to CCL22, but not monoclonal antibody to CCL17, significantly ( $P < 0.01$ ,  $n = 7$ –10 per group) decreased T<sub>reg</sub> cell migration into tumors, indicating that CCL22 has a role in tumor T<sub>reg</sub> cell migration. Identical treatment did not inhibit the migration of CD3<sup>+</sup>CD25<sup>+</sup> T cells into tumors (Fig. 4g). Therefore, CCL22 signal mediate the trafficking of T<sub>reg</sub> cells to tumors *in vivo*.

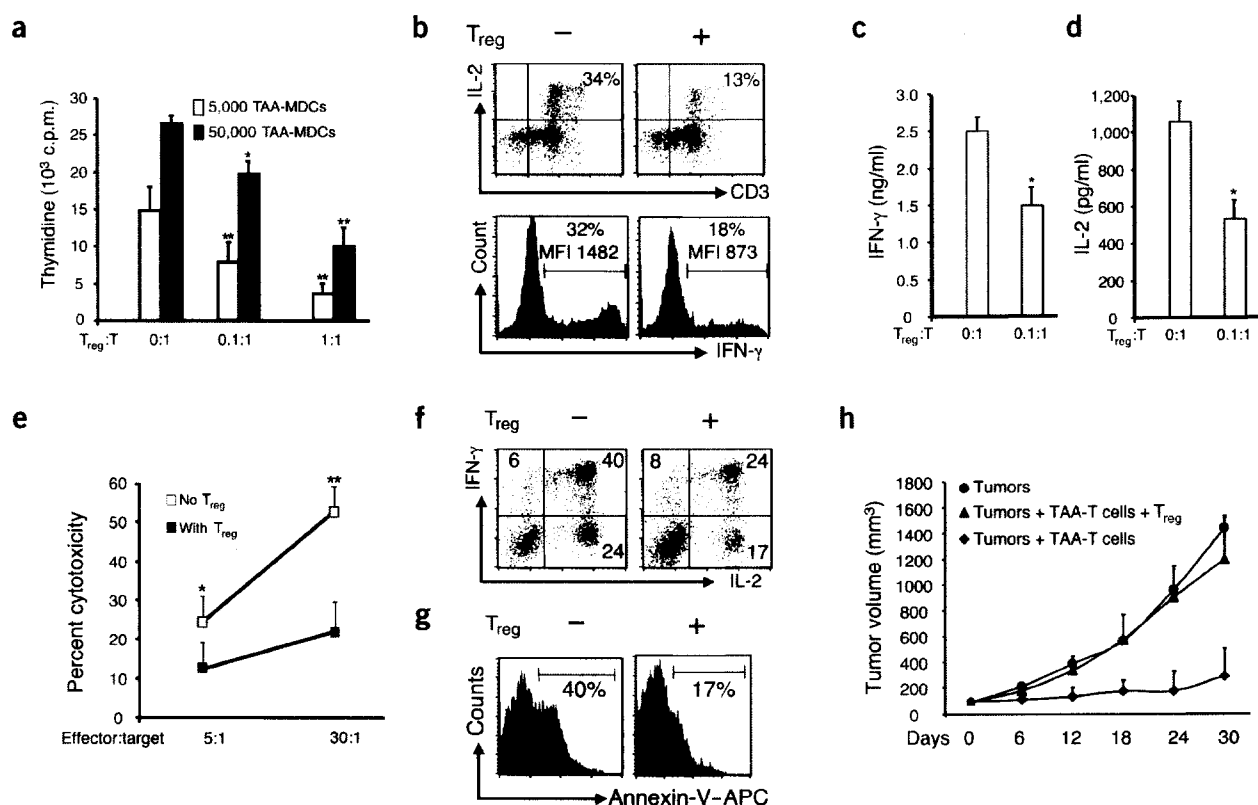
**Tumor T<sub>reg</sub> cells suppress Her2-specific immunity**

To determine the pathological importance of tumor T<sub>reg</sub> cells, we tested their TAA-specific suppressive functions *in vitro* and *in vivo*. We first used an *in vitro* system<sup>5,30</sup>. We obtained myeloid dendritic cells (MDCs)

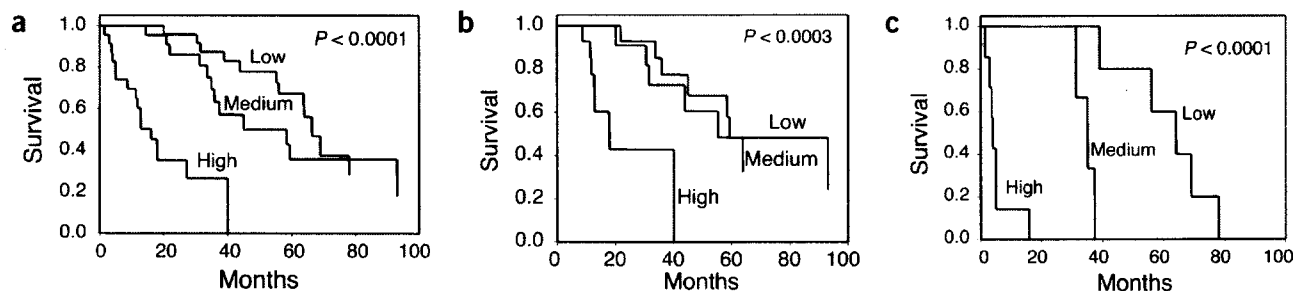
from HLA A2<sup>+</sup> individuals with tumors expressing Her2 (also known as Neu or ErbB2) and loaded them with Her2 peptides<sup>31</sup> to produce TAA-MDCs, which induced specific T cell activation as expected (Fig. 5). This induction of T cell proliferation was inhibited by autologous tumor ascites T<sub>reg</sub> cells ( $n = 5$ ,  $P < 0.05$ ; Fig. 5a).

Consistent with reports that strong T cell activation overcomes T<sub>reg</sub>-mediated suppression<sup>29,32</sup>, suppression was significantly more efficient when responder T cells were stimulated with fewer MDCs (Fig. 5a). Tumor T<sub>reg</sub> cells also blocked the production of IFN- $\gamma$  (Fig. 5b,c) and IL-2 (Fig. 5b,d) by effector T cells and Her2-specific cytotoxicity (Fig. 5e;  $P < 0.05$  for all). We observed similar and limited production of IL-4 and IL-10 with or without tumor T<sub>reg</sub> cells. TAA-MDCs stimulated poor proliferation of tumor T<sub>reg</sub> cells (<2,000 counts per min). MDCs without peptides stimulated poor T cell proliferation (<2,000 counts per min) and cytotoxicity (<10%).

To test the function of tumor T<sub>reg</sub> cells function *in vivo*, we stimulated tumor-associated T cells with autologous TAA-MDCs in



**Figure 5** Tumor T<sub>reg</sub> cells inhibit TAA-specific T cell immunity. (a) Tumor ascites T<sub>reg</sub> cells inhibit TAA-specific T cell proliferation *in vitro*. Tumor ascites CD3<sup>+</sup>CD25<sup>+</sup> T cells were stimulated with Her2 peptide-loaded MDCs (TAA-MDCs) for 6 d. Tumor T cell proliferation was detected by [<sup>3</sup>H]thymidine incorporation. Dendritic cells alone did not stimulate significant T cell proliferation ( $n = 5$ ,  $*P < 0.05$ ,  $**P < 0.01$ ). (b,c) Tumor T<sub>reg</sub> cells inhibit TAA-specific T cell production of IL-2 and IFN- $\gamma$  *in vitro*. Tumor T cells were stimulated with TAA-MDC in the presence of autologous tumor T<sub>reg</sub> cells. T cell cytokines were detected by intracellular staining (b) and ELISA (c,d) on day 6. (e) Tumor T<sub>reg</sub> cells inhibit Her2-specific T cell cytotoxicity *in vitro*. MDC-activated Her2-specific T cells were used as effector cells and Her2 peptide-loaded T2 cells were used as target cells. Her2-specific cytotoxicity was determined by FACS. Four HLA-A2<sup>+</sup> individuals were studied ( $*P < 0.05$ ,  $**P < 0.01$ ). (f,g) Tumor T<sub>reg</sub> cells suppress TAA-specific T cell immunity *in vivo*. Tumor ascites CD3<sup>+</sup> T cells plus TAA-MDCs were transferred into NOD/SCID mice with or without autologous tumor T<sub>reg</sub> cells. Three days after T cell transfer, either the peritoneal cells were collected and TAA-specific T cell IL-2 and IFN- $\gamma$  production was determined by FACS gated on human CD3<sup>+</sup> T cells (f), or HLA-A2<sup>+</sup> Her2<sup>+</sup> T2 cells were additionally injected into mice and T2 cells were collected from the peritoneal cavity after 40 h and analyzed for *in vivo* cytotoxicity by FACS (g). A representative experiment is shown ( $n = 4$ ,  $*P < 0.05$ ,  $**P < 0.01$ ). The T<sub>reg</sub>/CD3<sup>+</sup> T cell ratio was 0.1/1 in b–g.  $*P < 0.05$  versus no T<sub>reg</sub> cells in f and g. (h) Mice were injected with human primary ovarian tumors and treated with autologous tumor-specific T cells (group 2) or tumor-specific T cells plus tumor ascites T<sub>reg</sub> cells (group 3). Controls received no additional injection (group 1). Tumor volumes (mean  $\pm$  s.d.) were measured ( $n = 5$ –7 mice per group). The day of T<sub>reg</sub> cell injection was taken as day 0. At all time points after T cell injection,  $P < 0.05$  for group 3 or group 1 versus group 2.



**Figure 6** Accumulation of tumor  $T_{reg}$  cells predicts poor survival in individuals with ovarian carcinoma. A Kaplan-Meier curve for overall survival by number of tumor-infiltrating  $T_{reg}$  cells in 70 individuals with stages I–IV (a), stage III only (b) or stage IV only tumors (c). Samples were divided into three groups on the basis of the content of tumor-infiltrating  $T_{reg}$  cells. Survival significantly decreased as a function of  $T_{reg}$  cell content.

NOD/SCID mice using our xenotransplant model<sup>5</sup>. As expected and in support of the *in vitro* results, MDCs alone did not induce significant T cell activation (<5% IL-2<sup>+</sup> or IFN- $\gamma$ <sup>+</sup> T cells), whereas TAA-MDCs induced potent TAA-specific T cell production of IL-2 and IFN- $\gamma$  ( $P < 0.001$ ; Fig. 5f), and cytotoxic T lymphocyte (CTL) activity *in vivo* ( $P < 0.05$ ; Fig. 5g). We observed similar results when we used autologous primary ovarian tumor cells rather than Her2-pulsed T2 cells as the target cells. These TAA-specific immune responses were significantly inhibited *in vivo* by the adoptive transfer of autologous  $T_{reg}$  cells from tumor ascites (Fig. 5f,g).

Because tumor  $T_{reg}$  cells blocked tumor-specific T cell immunity *in vitro* and *in vivo* (Fig. 5a–g), we tested the hypothesis that tumor  $T_{reg}$  cells would disable TAA-specific T cell immunity *in vivo* and in turn allow tumor growth. Consistent with our previous observations<sup>5</sup>, mice without tumor-specific T cell transfusion showed progressive tumor growth ( $n = 7$ ), and mice with tumor-specific T cell transfusion showed significantly reduced tumor volume ( $n = 7$ ). Adoptively transferring tumor  $T_{reg}$  cells alone had no effects on tumor growth ( $n = 5$ ; data not shown). Mice receiving tumor-specific T cells plus tumor  $T_{reg}$  cells showed progressive tumor growth ( $n = 6$ ), comparable to controls without tumor-specific T cell transfer (Fig. 5h), indicating that tumor  $T_{reg}$  cells blocked the protective effects mediated by tumor-specific T cells. Taken together, these data indicate that tumor  $T_{reg}$  cells may hamper tumor-specific effector T cell immunity in individuals with cancer.

### Increases in tumor $T_{reg}$ cells predict poor survival

We predicted that tumor  $T_{reg}$  cells would adversely affect survival. To test this prediction, we analyzed all relevant clinical and pathological information, including tumor-associated survival (Supplementary Tables 1 and 2 online), that was available on 70 of our 104 individuals with cancer and correlated the data with the number of  $T_{reg}$  cells in tumor tissues determined by confocal microscopy. There was a significant correlation between tumor  $T_{reg}$  cell content and survival in the group as a whole ( $n = 70$ ,  $P < 0.0001$  for all), and also for individuals in stage II ( $P = 0.0362$ ), stage III ( $P = 0.0003$ ) and stage IV ( $P = 0.0001$ ; Supplementary Table 2 online). Tumor  $T_{reg}$  cells were a significant predictor of death hazard ( $P < 0.0001$ ), even after controlling for stage, surgical debulking and other factors known to affect survival, by using a Cox proportional hazards model.

In an alternative analysis, individuals were divided into three equal groups on the basis of numbers of tumor  $T_{reg}$  cells. The low  $T_{reg}$  cell group included all those with a tumor  $T_{reg}$  cell count of 131 or less per ten high-power field (HPF;  $n = 24$ ), the high  $T_{reg}$  cells group included those with a tumor  $T_{reg}$  cell count of 346 or more per ten HPF ( $n = 23$ ), and the medium tumor  $T_{reg}$  cell group included the remainder ( $n = 23$ ).

Survival functions were significantly different for the three groups ( $P < 0.0001$ ; Fig. 6a).

$T_{reg}$  cells were a significant predictor of death even after controlling for stage of disease and surgical debulking. Individuals in the highest  $T_{reg}$  cell group experienced a 25.1-fold higher death hazard as compared with those in the lowest  $T_{reg}$  cell group (95% confidence interval, 6.8–92.1). Individuals in the medium  $T_{reg}$  cell group experienced a 5.8-fold higher death hazard as compared with those in the lowest group (95% confidence interval, 1.9–17.5). Individuals in the highest  $T_{reg}$  cell group experienced a 4.2-fold reduction in survival, and those in the medium group experienced a 2.8-fold reduction in survival, as compared with those in the lowest  $T_{reg}$  cell group ( $P < 0.0001$ ; Fig. 6a).

The Cox proportional hazards model accounts for tumor stage, which is a known survival factor in ovarian cancer<sup>33</sup>. Nonetheless, we further stratified stage III and IV individuals into three subgroups of low ( $\leq 131$ ), medium (132–345) and high ( $\geq 346$ ) tumor  $T_{reg}$  cells numbers. Survival functions were still significantly different for the three groups in stage III ( $P < 0.0003$ ; Fig. 6b) and stage IV ( $P < 0.0001$ ; Fig. 6c). Therefore, an increase in the number of tumor  $T_{reg}$  cells is a significant predictor of increased risk for death and for reduced survival in ovarian cancer. This relationship holds whether  $T_{reg}$  cells are treated as a continuous or categorical variable (data not shown), and even after adjusting for disease stage and other significant clinical effects.

### DISCUSSION

Emerging evidence suggests that  $T_{reg}$  cells, particularly CD4<sup>+</sup>CD25<sup>+</sup>  $T_{reg}$  cells, are key mediators of peripheral tolerance<sup>15,16,28,34–36</sup>. Engendering strong antitumor immunity may thus involve breaking  $T_{reg}$ -mediated peripheral tolerance to TAAs. Consistent with this concept, experimental depletion of  $T_{reg}$  cells in mice with tumors improves immune-mediated tumor clearance<sup>37</sup> and enhances the response to immune-based therapy<sup>38</sup>.

Humans with cancer have increased numbers of peripherally circulating and tumor  $T_{reg}$  cells<sup>22–24</sup>. Previous studies have confirmed that these  $T_{reg}$  cells are functional through their inhibition of nonspecific T cell activation *in vitro*. Although this indirect evidence implicates  $T_{reg}$  cells in the immunopathogenesis of cancer, a definitive demonstration in humans is lacking. Our data show that  $T_{reg}$  cells in human ovarian cancers express intracellular CTLA-4, GITR and FOXP3, inhibit TAA-specific immunity *in vitro* and *in vivo*, and contribute to tumor growth. These data therefore provide direct *in vitro* and *in vivo* evidence that human  $T_{reg}$  cells have an important immunopathological role in human cancer by suppressing endogenous TAA-specific T cell immunity. We further linked this immunopathological role to clinical outcomes, by demonstrating that there is an inverse correlation between tumor  $T_{reg}$  cell content and patient survival.

Under homeostatic conditions, mouse and human T<sub>reg</sub> cells are found primarily in lymphoid organs<sup>14–17</sup>, a finding that our work confirms. On the basis of these observations, others have postulated that T<sub>reg</sub> cells mediate their suppressive effects by inhibiting T cell priming, which occurs in lymph nodes<sup>14,16,28</sup>. Nonetheless, mouse T<sub>reg</sub> cells also block antigen-specific T cell effector functions occurring outside lymph nodes<sup>39,40</sup>. In our studies of human ovarian carcinomas, there were significantly fewer T<sub>reg</sub> cells in tumor-draining lymph nodes than in control lymph nodes and tonsils. T<sub>reg</sub> cell numbers were also significantly lower in tumor-draining lymph nodes in late stage (III and IV) as compared with early stage (I and II) ovarian cancers. By contrast, there was a significant trend towards a higher accumulation of T<sub>reg</sub> cells in ascites and the solid tumor mass in later tumor stages.

Thus, T<sub>reg</sub> cells seem to migrate preferentially and predominantly to the tumor mass and the associated malignant ascites. This lack of apparent migration to lymph nodes suggests that tumor T<sub>reg</sub> cells may primarily work by inhibiting extranodal effector cell function rather than by suppressing naive T cell priming in lymph nodes, at least in later stages of tumors. This supposition is supported by our demonstration that tumor T<sub>reg</sub> cells inhibit effector T cell function *in vivo*, and this inhibition is associated with progressive tumor growth in the face of existing TAA-specific immunity.

Homeostatic and tumor T<sub>reg</sub> cells expressed similar amounts of the lymphoid homing molecules CCR7 and CD62L. It is unknown whether these molecules are involved in homeostatic T<sub>reg</sub> cell lymphoid homing. It is unclear why tumor T<sub>reg</sub> cells are not more abundant in draining lymph nodes. We favor the concept that T<sub>reg</sub> cell trafficking to tumor differs from the T<sub>reg</sub> cell trafficking to lymph nodes as previously shown for tumor plasmacytoid dendritic cells<sup>30</sup>. Consistent with this concept, normal blood T<sub>reg</sub> cells express CCR4 and CCR8, and migrate in response to CCL1, CCL17 or CCL22 on the basis of *in vitro* assays<sup>41</sup>. We have shown here, however, that tumor T<sub>reg</sub> cells express functional CCR4, the receptor for CCL22, and can migrate to CCL22 present in the tumor microenvironment.

Evidence suggests that CCL22 preferentially attracts activated antigen-specific T cells to dendritic cells<sup>42,43</sup>. Tumors may have capitalized on this effect to attract T<sub>reg</sub> cells to the microenvironment. Our data suggest that the source of this CCL22 is the ovarian tumor and associated macrophages. Functional tumor microenvironmental CCL22 has not been previously reported. Our studies show that blocking CCL22 *in vivo* reduces human T<sub>reg</sub> cell tumor trafficking. The data suggest that, in addition to depleting T<sub>reg</sub> cells, blocking T<sub>reg</sub> cell tumor trafficking represents a potential strategy for treating human cancers.

The accumulation of T<sub>reg</sub> cells in tumor predicts a marked reduction in patient survival, providing the 'smoking gun' that links T<sub>reg</sub> cells and the immunopathogenesis of human cancer. Taken together, these data provide the basis for developing novel immune-boosting strategies based on ridding the cancer patient of this cell population.

## METHODS

**Human samples.** We studied previously untreated individuals with epithelial ovarian carcinomas, classified as stage I to IV according to the International Federation of Gynecology and Obstetrics. Individuals gave written, informed consent. Specimens were collected at the Tulane University, New Orleans, Louisiana, USA, and the University of Turin, Turin, Italy. We obtained normal human tissues from the Cooperative Human Tissue Network. Histopathological findings were independently confirmed at the Tulane University, New Orleans, Louisiana, USA<sup>5</sup> and the University of Pennsylvania, Philadelphia, USA<sup>44</sup>. The study was approved by local institutional review boards.

**Cells and tissue biopsies.** Cells and tissues were obtained from ascites, blood, lymph nodes and tumors as described<sup>5,30,44</sup>. CD4<sup>+</sup> T cells were purified with

Untouched kits (Miltenyi). CD25<sup>+</sup> cells were purified with paramagnetic beads (Miltenyi) and sorted with phycoerythrin (PE)-conjugated antibody to CD25 (PharMingen)<sup>21</sup>. Cell populations were assessed to be more than 90% pure by FACS.

**FACS and PCR.** Details of FACS and PCR<sup>44–46</sup> are given in **Supplementary Methods** online.

**CD4<sup>+</sup>CD25<sup>+</sup> T cells and FOXP3<sup>+</sup> T cells in human tissues.** Detailed information is given in **Supplementary Methods** online.

***In vitro* non-antigen-specific immunosuppression.** Tumor ascites CD3<sup>+</sup>CD25<sup>−</sup> T cells (10<sup>5</sup> per ml) were stimulated with soluble monoclonal antibody to human CD3 (5 μg/ml, UCHT, IgG1; PharMingen) and monoclonal antibody to human CD28 (2.5 μg/ml, cd28.2, IgG1; PharMingen) for 6 d. The added autologous CD4<sup>+</sup>CD25<sup>+</sup> T cell/CD3<sup>+</sup>CD25<sup>−</sup> T cell ratio was 0/1, 0.1/1 or 1/1. On day 6, T cell proliferation and cytokines were detected as described<sup>5,30</sup>.

**Migration and adhesion or transmigration.** Migration and adhesion or transmigration were assessed as described<sup>30</sup> using 5–20 × 10<sup>4</sup> CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells. Human chemokines (CXCL12, CCL17, CCL19, CCL21 and CCL22, 100 ng/ml of each; all from R&D Systems), or cell-free tumor ascites were added to the lower chamber. Antibodies to CCL17 (540.26, IgG1, 500 ng/ml), CCL22 (57226.11, IgG2b, 500 ng/ml) and CXCR4 (44717, IgG2b, 500 ng/ml) were from R&D Systems. We confirmed the identity of migrating T<sub>reg</sub> cells by FACS analysis for CD4 and CD25.

**CCL22 protein.** CCL22 protein was detected by ELISA (R&D Systems). Intracellular human CCL22 was detected by FACS with monoclonal antibody to CCL22 (57226.11, IgG2b, R&D Systems), counterstained with fluorescein isothiocyanate (FITC)-conjugated goat antibody to mouse IgG (PharMingen).

***In vivo* migration.** Human primary ovarian epithelial carcinomas were established in female NOD.CB17/SCID<sup>5</sup> mice aged 6–8 weeks (Jackson Laboratories). On day 12 after tumor inoculation, human CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells or CD3<sup>+</sup>CD25<sup>−</sup> T cells (2.5 × 10<sup>6</sup>) were injected in a 100-μl volume into tail veins. Some mice received intraperitoneal mouse antibody to human CCL22 (500 ng per 200 μl, 57226.11, IgG2b; R&D Systems), or control IgG (PharMingen), 48 h before and 24 h after T cell injection. After 48 h, some mice were killed and the extracted tumors were mechanically disrupted. Human CD3<sup>+</sup> T cells were identified by FACS with antibody to human CD3 and are expressed as human CD3<sup>+</sup> T cells per 10<sup>8</sup> tumor cells.

***In vitro* TAA-specific immunosuppression.** Tumor MDCs were differentiated from tumor macrophages as described<sup>47</sup>. Dendritic cells were loaded with 5 μg/ml of three distinct HLA-A2-binding Her2 peptides<sup>31</sup>, p369–384 (KIFGSLAFLPESFDGDP), p688–703 (RRLLQETELVEPLTPS) and p971–984 (ELVSEFSRMARDPQ), or with an HLA-A2 influenza virus matrix control peptide GILGFVFTL (Multiple Peptide System). Peptide-loaded MDCs (TAA-MDCs, 5–50 × 10<sup>3</sup> per ml) were used to activate autologous tumor CD3<sup>+</sup>CD25<sup>−</sup> cells (10<sup>5</sup> per ml). The added T<sub>reg</sub>/CD3<sup>+</sup>CD25<sup>−</sup> T ratio was 0/1, 0.1/1 or 1/1. On day 6, we detected TAA-specific T cell proliferation and cytokines as described<sup>5,30</sup>. T2 cells (HLA-A2<sup>+</sup> lymphoblastoid cell lines; 5 × 10<sup>6</sup> per ml, American Type Culture Collection) were labeled with 10 μM 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) for 10 min in the dark at 37 °C. Cytotoxicity was assessed by using CFSE-labeled T2 cells bearing all three Her2 peptides or the matrix control with annexin V and 7-AAD staining<sup>48</sup>.

***In vivo* TAA-specific T cell immunosuppression.** We used cells from ascites of HLA-A2<sup>+</sup> individuals affected with Her2<sup>+</sup> ovarian cancer. TAA-MDCs (10<sup>6</sup> per mouse) and autologous tumor ascites CD3<sup>+</sup>CD25<sup>−</sup> T cells (10<sup>7</sup> per mouse) were injected into NOD/SCID mice with or without autologous tumor T<sub>reg</sub> cells (10<sup>6</sup> per mouse). After 3 d, we collected the peritoneal cells and assayed them for intracellular IL-2 and IFN-γ gated on human CD3<sup>+</sup> T cells as described<sup>5,30</sup>. In some experiments, 3 d after the first injection the mice were injected intraperitoneally with Her2<sup>+</sup>, HLA-A2<sup>+</sup>, CFSE-labeled T2 cells or autologous primary ovarian

tumor cells ( $10^8$  per mouse). Forty hours after this second injection, we collected the T2 cells and stained them with annexin V and 7-AAD to determine cytotoxicity<sup>48</sup>. All studies were approved by the Tulane Animal Use Committee.

**In vivo tumor regression assay.** Primary ovarian tumor cells ( $1 \times 10^7$ ) in 200  $\mu$ l of buffered saline were injected into dorsal subcutaneous tissues<sup>5</sup>. Autologous tumor ascites CD4<sup>+</sup>CD25<sup>+</sup>CD3<sup>+</sup> cells ( $2.5 \times 10^6$ ) were injected intravenously into mice in 100  $\mu$ l of buffered saline on day 12 after human tumor inoculation. In some cases, 12 h after injecting tumor ascites CD4<sup>+</sup>CD25<sup>+</sup>CD3<sup>+</sup> cells, tumor-specific T cells ( $5 \times 10^6$ ) were injected intravenously into mice. Tumor size was measured twice weekly using calipers fitted with a Vernier scale. Tumor volume was calculated on the basis of three perpendicular measurements<sup>5</sup>.

**Statistical analysis.** Details of all statistical analyses are given in **Supplementary Methods** online.

*Note: Supplementary information is available on the Nature Medicine website.*

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#### COMPETING INTERESTS STATEMENT

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# Bone Marrow Is a Reservoir for CD4<sup>+</sup>CD25<sup>+</sup> Regulatory T Cells that Traffic through CXCL12/CXCR4 Signals

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## ABSTRACT

CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Tregs) mediate peripheral T-cell homeostasis and contribute to self-tolerance. Their homeostatic and pathologic trafficking is poorly understood. Under homeostatic conditions, we show a relatively high prevalence of functional Tregs in human bone marrow. Bone marrow strongly expresses functional stromal-derived factor (CXCL12), the ligand for CXCR4. Human Tregs traffic to and are retained in bone marrow through CXCR4/CXCL12 signals as shown in chimeric nonobese diabetic/severe combined immunodeficient mice. Granulocyte colony-stimulating factor (G-CSF) reduces human bone marrow CXCL12 expression *in vivo*, associated with mobilization of marrow Tregs to peripheral blood in human volunteers. These findings show a mechanism for homeostatic Treg trafficking and indicate that bone marrow is a significant reservoir for Tregs. These data also suggest a novel mechanism explaining reduced acute graft-versus-host disease and improvement in autoimmune diseases following G-CSF treatment.

## INTRODUCTION

CD4<sup>+</sup>CD25<sup>+</sup> T cells (Tregs) mediate peripheral T-cell homeostasis (1–3). Studies in murine models show that CD4<sup>+</sup>CD25<sup>+</sup> T cells are essential for the induction of tolerance to alloantigens and inhibit graft-versus-host disease (GVHD; refs. 4, 5). Human blood Tregs express CCR4 and CCR8 and migrate in response to their ligands *in vitro* (6). We recently showed that pathologic Tregs in human ovarian cancers migrate into tumor in response to CCL22 (7). Homeostatic Tregs may originate in the thymus, although their differentiation in the periphery also has been suggested. Despite these recent advances in our knowledge, relatively little is known regarding the natural reservoirs of migrating Tregs or signals that induce their mobilization of trafficking.

Bone marrow is vascularized by blood but not by lymphatic vessels. Bone marrow is a part of the lymphocyte recirculation network (8), with billions of lymphocytes recirculating through it each day. We hypothesized that bone marrow might harbor CD4<sup>+</sup>CD25<sup>+</sup> Tregs and function as a reservoir for them. In this capacity, it could be an important organ to fine tune T-cell immunity by modulating Treg trafficking. We further hypothesized that granulocyte colony-stimulating factor (G-CSF) would mobilize bone marrow CD4<sup>+</sup>CD25<sup>+</sup> Tregs. We tested these hypotheses in defined models and human subjects and now show a high prevalence of functional Tregs in human bone marrow. We provide evidence that CXCR4/CXCL12 signals play an important role in regulating Treg egress from bone marrow and in maintaining homeostatic levels of Tregs in the periphery. G-CSF mobilizes Tregs from bone marrow into the periphery by decreasing marrow CXCL12 expression.

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These data may be used to manipulate Tregs for therapeutic purposes and may help to explain the low prevalence of acute GVHD in recipients of G-CSF-mobilized bone marrow transplants (9, 10) and the improvement in autoimmune diseases following G-CSF treatment (11, 12).

## MATERIALS AND METHODS

**Human Subjects.** Written, informed consent was obtained for all of the subjects. The Institutional Review Board of Tulane Medical School approved the study. Healthy adults were studied untreated or immediately following subcutaneous injection of 5 µg/kg recombinant human G-CSF (Filgrastim; Amgen Inc., Thousand Oaks, CA) once daily for four consecutive days. Peripheral blood cells, bone marrow cells, and bone marrow fluid (cell-free bone marrow) were collected and frozen for later use. Human thymus and tonsils were collected from young children undergoing cardiac surgery or other treatments and mechanically disrupted into single cell suspensions. Cells were stained with monoclonal antibodies and analyzed on a FACSCalibur (Becton-Dickinson, Franklin Lakes, NJ) using mouse antihuman CD4-FITC (SK3, IgG1) and mouse antihuman CD25-phycoerythrin (MA251, IgG1; all from BD PharMingen, San Diego, CA).

**Mice.** The study was approved by the Institutional Animal Care and Use Committee of the Tulane Medical School. Six- to 8-week-old female C57/black 6 mice were used (Jackson Labs, Bar Harbor, ME). Peripheral blood was collected by cardiac aspiration into heparinized glass tubes. Spleen and inguinal lymph nodes were mechanically disrupted into single cell suspensions. Bone marrow was collected from the femur, tibia, and humerus by flushing with 200 µL sterile PBS. After centrifugation, the supernatant (bone marrow fluid) was frozen for later detection of chemokines and for migration assays. CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> T cells in blood, spleen, and lymph nodes were identified with fluorescence-activated cell sorting (FACS) using antimouse CD4 (clone RM 4–5; eBioscience, San Diego, CA), CD25 (PC61; eBioscience), and CD3 (145–2c11; BD PharMingen). At least 5000 gated events per condition were analyzed using CellQuest software (Becton-Dickinson).

**Reverse Transcription-PCR.** Real-time reverse transcription-PCR was carried out for *FOXP3* using primers for upstream 5'-cagctgccacactgccctag-3', downstream 5'-cattgccagcagtggttag-3', and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). cDNA was against normalized *GAPDH* and expressed as fold difference relative to *GAPDH* (13).

**Immunosuppression Assay.** Human bone marrow CD4<sup>+</sup> T cells were purified with Untouched kits (Miltenyi, Auburn, CA). CD25<sup>+</sup> cells were sorted with anti-CD25-phycoerythrin (BD PharMingen). Cell populations were >90% pure by flow cytometry. Monocyte-derived dendritic cells (MDCs) were differentiated as we described previously (14). Dendritic cells were incubated with HLA-A2-binding influenza virus peptide GILGFVFTL (Multiple Peptide System, Seattle, WA), and 4 to 20 × 10<sup>3</sup>/mL MDCs were used to activate autologous CD3<sup>+</sup> T cells (10<sup>5</sup>/mL). The Treg to CD3<sup>+</sup> T-cell ratio was 0:1, 0.5:1, or 1:1 as indicated. On day 6, antigen-specific T-cell proliferation and cytokines were detected as we described previously (15, 16).

**In vitro Migration Assay and In vitro Adhesion/Transmigration Assay.** Migration and transmigration were assessed as we described previously (15) using human CD4<sup>+</sup>CD25<sup>+</sup> Tregs (5 to 20 × 10<sup>4</sup>). Tregs were induced to migrate with recombinant human CXCL12 (100 ng/mL; R&D Systems, Minneapolis, MN) or human bone marrow fluid or mouse bone marrow fluid. Tregs were incubated with mouse antihuman CXCR4 (44717, IgG2b; 500 ng/mL) for 2 hours as indicated. Identity of migrating Tregs was further confirmed using FACS for CD3, CD4, and CD25.

CXCL12 protein in bone marrow fluids was detected by ELISA (R&D Systems).

**In vivo Migration.** Human blood  $CD4^+CD25^+$  T cells ( $2.5 \times 10^6$  in 200  $\mu$ L PBS) were injected in 100  $\mu$ L volume into tail veins in female NOD.CB17-SCID mice (6 to 8 weeks old; Jackson Labs; ref. 16). Mice were injected intraperitoneally two times with mouse antihuman CXCR4 (500 ng/200  $\mu$ L; 44717, IgG2b; R&D Systems) or control antibody (IgG; BD Pharmingen) 12 hours before and 12 hours after Treg injection. Twenty to 60 hours later, animals were killed, and bone marrow, blood, and spleens were collected. Human  $CD3^+$  T cells were identified using FACS with antihuman- $CD3$  antibody, expressed as human  $CD3^+$  T cells per  $10^6$  mononuclear cells.

**Statistical Analysis.** Differences in cell surface molecule expression were determined by  $\chi^2$  test and in other variables by unpaired  $t$  test with  $P < 0.05$  considered significant.

## RESULTS

**$CD4^+CD25^+FOXP3^+$  T Cells in Human Bone Marrow.** Homeostatic  $CD4^+$  Tregs (identified as  $CD4^+CD25^+$  T cells) are found in peripheral blood and lymphoid organs (2, 3, 17, 18). Recent reports suggest that bone marrow is a site for important T-cell immune events (19–21). Thus, we examined bone marrow for the presence of  $CD4^+CD25^+$  T cells. Flow cytometric analysis (FACS) showed the presence of  $CD3^+CD4^+CD25^+$  T cells in the bone marrow of healthy human donors (Fig. 1A). The fraction of  $CD3^+CD4^+CD25^+$  T cells among all of the  $CD4^+CD3^+$  T cells (Fig. 1A) or all of the nucleated cells (Fig. 1B) was significantly higher in human bone marrow than in blood, lymph nodes, or thymus ( $P < 0.01$  for each).

The expression of *FOXP3* is correlated with regulatory activity of human blood Tregs (22). Reverse transcription-PCR showed that the expression of *FOXP3* was higher in bone marrow  $CD4^+CD25^+$  T cells than in blood  $CD4^+CD25^+$  T cells ( $P < 0.05$ ; Fig. 1C).  $CD4^+CD25^{high}$  Tregs were reportedly to be more suppressive than  $CD4^+CD25^{low}$  Tregs (23). Multicolor staining revealed that the frac-

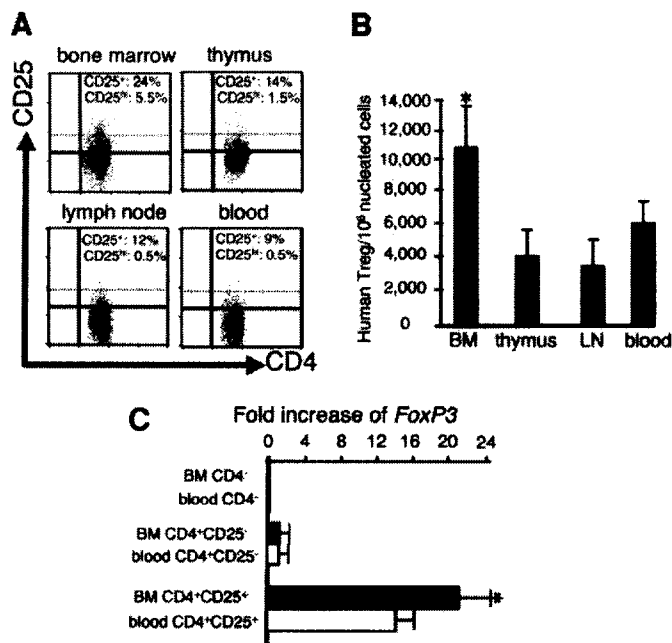


Fig. 1.  $CD3^+CD4^+CD25^+FOXP3^+$  cells in bone marrow. A.  $CD3^+CD4^+CD25^+$  cells in human bone marrow. FACS showed a large proportion of  $CD4^+CD25^+$  T cells in bone marrow in healthy human donors. Cells were analyzed with multiple color staining and gated on  $CD3^+CD4^+$  cells. Results are expressed as the percentage of  $CD3^+CD4^+CD25^+$  cells in  $CD3^+CD4^+$  cells ( $n = 7$ ). B. Human  $CD3^+CD4^+CD25^+$  cells were identified by FACS and expressed as mean  $\pm$  SE of  $CD4^+CD25^+$  T cells per  $10^6$  nucleated cells ( $n = 7$ ;  $*P < 0.01$ , compared with thymus, lymph nodes, and blood). C. *FOXP3* was detected by reverse transcription-PCR in the indicated cells ( $n = 6$ ;  $*P < 0.001$ , compared with  $CD4^-$  cells or  $CD4^+CD25^-$  cells;  $P < 0.05$  for bone marrow  $CD4^+CD25^+$  compared with blood  $CD4^+CD25^+$  cells).

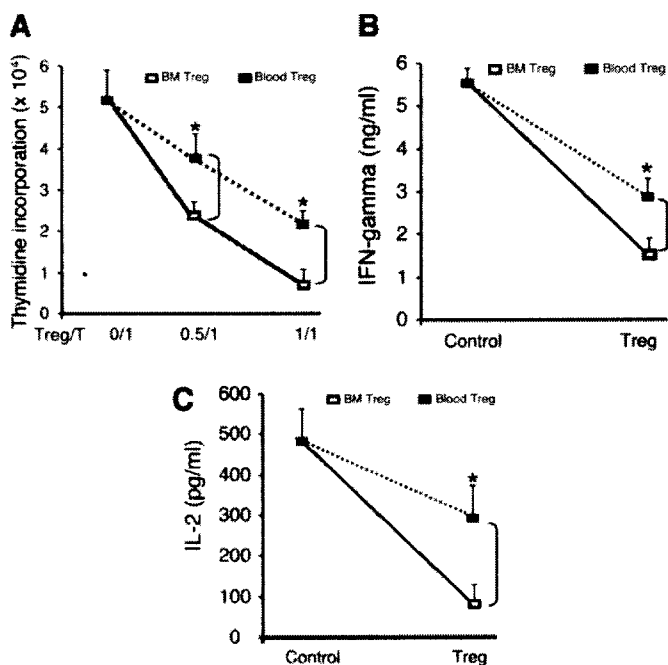


Fig. 2. Functional  $CD4^+CD25^+$  Tregs in bone marrow.  $CD3^+$  T cells were stimulated with antigen-loaded MDCs for 6 days with or without bone marrow  $CD3^+CD4^+CD25^+$  cells (Tregs). A. Human bone marrow Tregs inhibited MDC-mediated T-cell proliferation detected by [ $^3$ H]thymidine incorporation. B. Human bone marrow Tregs inhibited T-cell  $\gamma$ -interferon production. C. Human bone marrow Tregs inhibited T-cell interleukin 2 production ( $n = 6$ ;  $*P < 0.01$  for blood Tregs compared with bone marrow;  $P < 0.001$  for blood marrow Tregs and blood Tregs compared with no Tregs). LN, lymph nodes; BM, bone marrow. Treg to responder T cells = 1:1 for B and C.

tion of  $CD4^+CD25^{high}$  T cells was higher in bone marrow ( $5.5 \pm 3.5\%$ ;  $n = 7$ ) than in thymus ( $1.5 \pm 1.2\%$ ;  $n = 7$ ), lymph nodes ( $0.5 \pm 0.3\%$ ;  $n = 9$ ), or blood ( $0.5 \pm 0.4\%$ ;  $n = 12$ ;  $P < 0.01$  for each; Fig. 1A). Thus, bone marrow harbored a significant population of  $CD4^+CD25^+FOXP3^+$  T cells. We hypothesized that these bone marrow  $CD4^+CD25^+FOXP3^+$  T cells were functional regulatory T cells as described for such cells in blood (3) and other compartments (7).

**Bone Marrow  $CD4^+CD25^+FOXP3^+$  T Cells Are Functional Regulatory T Cells.** To test whether human bone marrow  $CD4^+CD25^+$  T cells are functional regulatory T cells, we used our antigen-specific T-cell culture system (15, 16). Myeloid dendritic cells pulsed with influenza peptides induced antigen-specific T-cell activation as expected (Fig. 2A–C). Inclusion of Tregs purified from autologous bone marrow significantly inhibited antigen-specific T-cell proliferation (Fig. 2A),  $\gamma$ -interferon, and interleukin 2 production (Fig. 2B and C) in a dose-dependent manner ( $n = 5$ ;  $P < 0.05$ ). Strikingly, bone marrow  $CD4^+CD25^+$  T cells were significantly more potent than blood  $CD4^+CD25^+$  T cells in inhibiting T-cell activation ( $n = 5$ ;  $P < 0.05$  for all; Fig. 2A–C). Thus,  $CD3^+CD4^+CD25^+$  T cells in bone marrow are functional regulatory T cells (Tregs). Human bone marrow Tregs were superior to blood counterparts in suppressing T-cell activation (Fig. 2A–C) and expressed more *FOXP3* than their blood counterparts (Fig. 1C). The data suggest that bone marrow Tregs may be activated and/or memory regulatory T cells.

**Activated  $CD4^+CD25^+$  Regulatory T Cells Migrate with Bone Marrow-derived CXCL12.** We next examined whether Tregs trafficked into bone marrow. In preliminary *in vitro* chemotaxis assays, freshly isolated blood  $CD3^+CD4^+CD25^+$  Tregs did not efficiently migrate in response to bone marrow fluid (not shown). Because Tregs in bone marrow expressed an apparently activated phenotype (Fig.

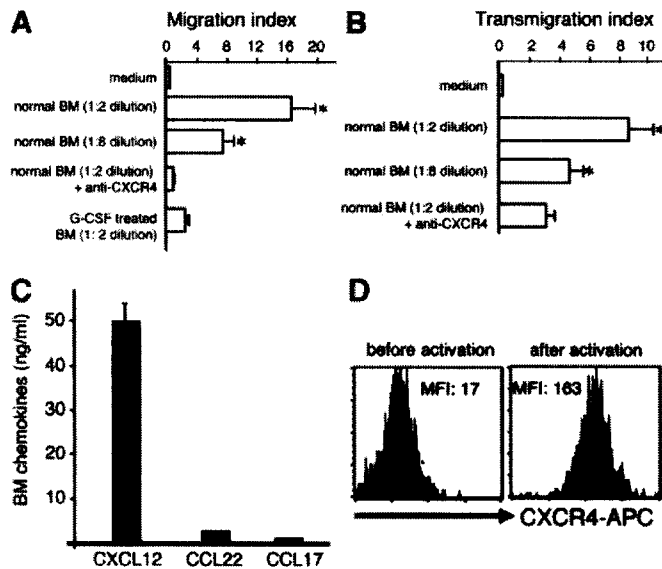


Fig. 3. Activated Tregs migrate toward bone marrow through CXCR4/CXCL12 signals *in vitro*. MDC-activated blood Tregs migrated toward human bone marrow (BM) fluid. **A**, *in vitro* chemotaxis assay. Activated blood Tregs migrated toward human bone marrow fluid obtained from normal donors or G-CSF-treated normal donors. **B**, *in vitro* transmigration assay. Activated blood Tregs transmigrated toward human bone marrow fluid from normal donors ( $n = 6$ ;  $*P < 0.01$  compared with medium or anti-CXCR4 for **A** and **B**). **C**, Human bone marrow fluid contained high level of CXCL12 detected by ELISA ( $n = 8$ ). **D**, Activated blood Tregs expressed high-level CXCR4 gated on CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> cells. One of four representative experiments is shown.

1C, Fig. 2). we activated human blood CD4<sup>+</sup>CD25<sup>+</sup> T cells with allogeneic MDCs for 48 hours to replicate this activated state. Now MDC-activated blood Tregs efficiently migrated in response to human bone marrow fluid in the *in vitro* chemotaxis assay and in the *in vitro* adhesion and transmigration assay ( $P < 0.01$ ) in a dose-dependent manner (Fig. 3A and B). Antihuman CXCR4 significantly ( $P < 0.05$ ) decreased bone marrow fluid-mediated Treg migration (Fig. 3A and B). In support, we showed that human bone marrow fluid contained a high level of CXCL12 (the sole CXCR4 ligand) but not CCL22 or CCL17 (Fig. 3C), chemokines shown to mediate blood Treg migration *in vitro* (6). In further support, MDC activation increased CXCR4 expression on CD4<sup>+</sup>CD25<sup>+</sup> T cells (Fig. 3D). Therefore, human bone marrow mediates Treg migration and local accumulation by CXCR4/CXCL12 signals *in vitro*.

**CD4<sup>+</sup>CD25<sup>+</sup> Regulatory T Cells Traffic into Bone Marrow through CXCR4/CXCL12 Signals.** Having shown a role for CXCL12/CXCR4 signals *in vitro*, we next addressed the role for this axis *in vivo* in our human–nonobese diabetic/severe combined immunodeficient (NOD/SCID) chimeric mouse model (16). Forty to 60 hours after intravenous human Treg transfusion, human Tregs were primarily found in bone marrow. Far fewer Tregs were found in spleen (Fig. 4A), and  $<100$  cells/mL were detected in peripheral blood. Strikingly, *in vivo* administration of a specific antihuman CXCR4 monoclonal antibody significantly ( $P < 0.05$ ) decreased Treg migration into bone marrow but not into spleen (Fig. 4B). We further showed that mouse bone marrow produced high level of CXCL12 but not CCL22 or CCL17 (Fig. 4C). In further support, mouse bone marrow efficiently mediated human MDC-activated Treg migration *in vitro* in a dose-dependent manner. This *in vitro* migration was significantly blocked by mouse antihuman CXCR4 monoclonal antibody (Fig. 4D). Collectively, these data indicate that CXCL12/CXCR4 signals are critical for Treg trafficking to bone marrow *in vivo*.

**G-CSF Mobilizes Bone Marrow CD4<sup>+</sup>CD25<sup>+</sup> Regulatory T Cells through Reducing CXCL12.** After determining that Tregs would migrate to and remain in bone marrow, we next examined

whether bone marrow Tregs would have the potential to traffic back into the periphery. G-CSF mobilizes mouse hematopoietic stem cells and neutrophils by disrupting the CXCR4/CXCL12 chemotactic interaction (24, 25). Human bone marrow also expresses significant quantities of the chemokine CXCL12 (Fig. 3C). We hypothesized that G-CSF would mobilize human bone marrow Tregs. Consistent with our hypothesis, G-CSF treatment of healthy human volunteers significantly ( $P < 0.01$ ) decreased Tregs in their bone marrow and significantly ( $P < 0.05$ ) increased Treg in their peripheral blood (Fig. 5A and B). In support, we showed that G-CSF treatment significantly reduced bone marrow CXCL12 expression in healthy human volunteers (Fig. 5C). Bone marrow from G-CSF-treated volunteers was significantly less efficient in mediating Treg migration than bone marrow from control subjects (Fig. 3A;  $P < 0.001$ , compared with normal bone marrow). These data suggested that G-CSF mobilizes Tregs from bone marrow through disruption of CXCL12/CXCR4 signals *in vivo* and that bone marrow Tregs can traffic into the periphery.

## DISCUSSION

Homeostatic Tregs mediate peripheral tolerance to self-antigens by suppressing autoreactive immune cells. Emerging evidence implicates regulatory T cells, particularly CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Tregs) in the pathogenesis of autoimmune diseases, tumors, and organ transplantation (2, 3, 17, 18, 26). Classic, natural CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells are thought to reside primarily in lymphoid organs (2, 3, 17, 18). On the basis of these observations, it is postulated that Tregs mediate their suppressive effects by inhibiting T-cell priming, which occurs in lymph nodes. Bone marrow is vascularized by blood but not by lymphatic vessels. Bone marrow is a part of the lymphocyte recirculation network. Billions of lymphocytes circulate through bone marrow each day (8). We found large numbers of functional Tregs in

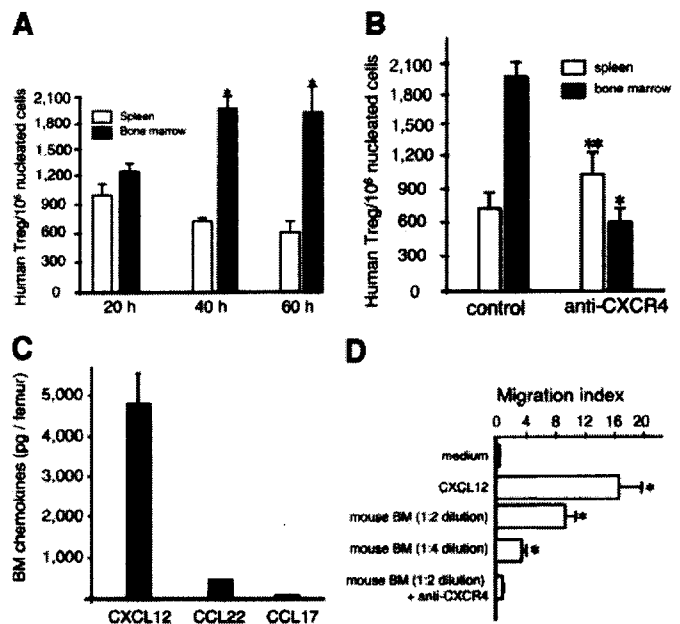


Fig. 4. Activated Tregs migrate toward bone marrow through CXCR4/CXCL12 signals *in vivo*. **A**, Human Tregs trafficked to bone marrow. Human Tregs were transferred intravenously into NOD/SCID mice. Twenty, 40, and 60 hours after Treg transfusion, human T cells were identified by FACS (mean  $\pm$  SD;  $n = 6$ ;  $*P < 0.01$  compared with spleen). **B**, Blocking CXCR4 (see Materials and Methods) decreased human Treg bone marrow trafficking ( $*P < 0.01$ ;  $**P < 0.05$  compared with controls). **C**, CXCL12 was detected in mouse bone marrow using ELISA ( $n = 8$ ). **D**, Mouse bone marrow-mediated Treg migration was evaluated in an *in vitro* migration assay (mean  $\pm$  SD;  $n = 6$ ;  $*P < 0.01$ , compared with medium or anti-CXCR4 antibody).

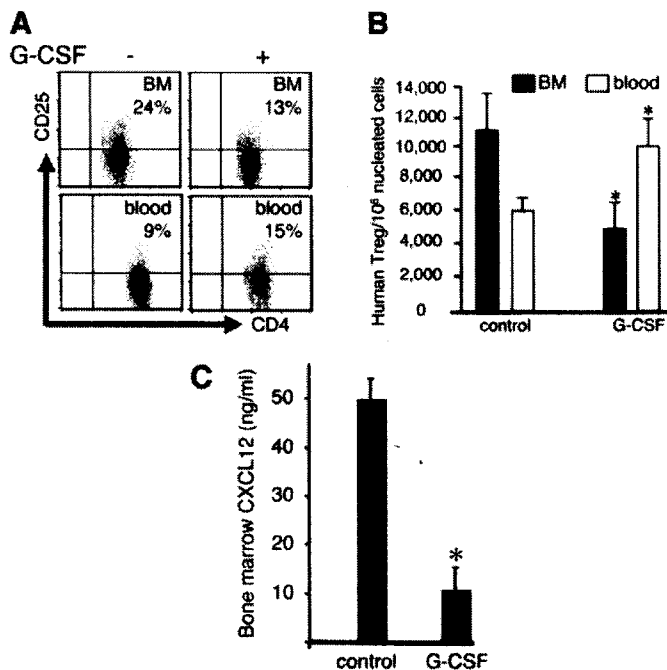


Fig. 5. G-CSF mobilizes human bone marrow Tregs by interrupting CXCR4/CXCL12 signals. G-CSF treatment reduced Treg content in human bone marrow and increased Treg numbers in blood. A. The percentage of Treg in CD4<sup>+</sup> T cells (FACS) is shown. B. Absolute numbers of Tregs per 10<sup>6</sup> total nucleated cells were shown. C. G-CSF administration reduced CXCL12 in human bone marrow. CXCL12 in bone marrow fluid was detected using ELISA ( $n = 7$ ; \* $P < 0.01$  for all, compared with control).

bone marrow. This finding provides evidence for a previously unidentified role of bone marrow in T-cell homeostasis: Bone marrow is a preferential site for migration or selective retention and function of Tregs. The study significantly complements recent reports that bone marrow harbors antigen-specific memory T cells (20) and is an important site for T-cell priming (19).

Conventional lymphocyte trafficking has been extensively investigated (27). However, little is known regarding the natural reservoirs for migrating Tregs and the trafficking signals for human Tregs. Our study is the first to show a mechanism for human Treg homeostatic trafficking *in vivo*.

We also suggest that bone marrow is a significant reservoir for human Tregs and that CXCL12/CXCR4 signals are critical for Treg trafficking between bone marrow and periphery. Several lines of evidence support this notion. First, CXCL12 is expressed in marrow and induces Treg chemotaxis and adhesion/transmigration. Second, G-CSF mobilizes human bone marrow Tregs through reducing bone marrow-derived CXCL12. Third, human Treg preferentially home to bone marrow but not to spleen under homeostatic conditions in NOD/SCID mice. Finally, blocking CXCL12/CXCR4 signals significantly reduces Treg trafficking to bone marrow. There is one amino acid difference between mouse and human CXCL12 (28, 29). Thus, our work also suggests that this human-NOD/SCID chimeric model is relevant to study the role of CXCL12/CXCR4 signals in human Treg trafficking.

Expression of *FOXP3* can be induced by activation and is associated with the suppressive capacity of Tregs (22). Activated Tregs are more efficient in blocking T-cell activation than nonactivated Tregs (30). Here we show that bone marrow Tregs express more *FOXP3* and CD25 than blood counterparts and are functionally superior to their blood counterparts in suppression. We also show that MDC activation of blood Tregs augments their CXCR4 expression, which significantly enhances their migration toward bone marrow-derived CXCL12.

Thus, we suggest that bone marrow Tregs contain "memory" and/or activated Tregs.

We showed that human ovarian tumors produce high-level CXCL12 that mediates tumor-associated plasmacytoid dendritic cell trafficking (15). Although we recently showed that there are significant numbers of functional, tumor-infiltrating Tregs in human ovarian tumors, tumor environmental CCL22 but not CXCL12 is crucial for this Treg tumor trafficking *in vivo* (7). Thus, local tumor microenvironmental factors may account for these migratory differences. Alternatively, there may be distinct Treg subsets specifically recruited in response to selected stimuli. In either regard, our data suggest that Treg distribution and trafficking may be regulated in a tissue- and/or organ-specific manner that is further subject to modification by local environmental factors.

Agents that mobilize Tregs from bone marrow could be therapeutically beneficial in some clinical settings. We showed here that G-CSF decreases bone marrow CXCL12 and in turn mobilizes bone marrow Tregs. These findings may help explain how G-CSF administration reduces the severity and mortality in acute GVHD (9, 10). In support, mouse Tregs reduce the severity of GVHD (5, 31). G-CSF also ameliorates the autoimmune diseases systemic lupus erythematosus and experimental autoimmune allergic encephalomyelitis in mouse models (11, 12).

Administration of anti-CXCL12 counteracts B1a-lymphocyte expansion and T-lymphocyte activation and decreases autoantibody production and nephritis in murine lupus (32). The CXCR4 antagonist AMD3100 also inhibits autoimmune collagen-induced arthritis in mice (33). These findings may result from Treg mobilization through interrupting CXCR4/CXCL12 signals in bone marrow and suggest additional means for Treg mobilization.

Our data show that recruitment of Tregs into bone marrow through CXCL12 represents a novel and important mechanism of Treg homeostatic traffic. Mobilizing bone marrow Tregs may be a novel strategy to manipulate systemic immunity therapeutically.

## ACKNOWLEDGMENTS

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## Dendritic Cell Subsets Differentially Regulate Angiogenesis in Human Ovarian Cancer

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### Abstract

Angiogenesis is essential for both primary and metastatic tumor growth. Tumor blood vessel formation is complex and regulated by many factors. Ovarian carcinomas have a poor prognosis, often associated with multifocal intraperitoneal dissemination accompanied by intense neovascularization. To examine tumor angiogenesis in the tumor microenvironment, we studied malignant ascites of patients with untreated ovarian carcinoma. We observed high numbers of plasmacytoid dendritic cells (PDCs) and significant stromal-derived factor (CXCL-12/SDF)-1 in their malignant ascites, attracting PDCs into the tumor environment. We now show that tumor-associated PDCs induced angiogenesis *in vivo* through production of tumor necrosis factor  $\alpha$  and interleukin 8. By contrast, myeloid dendritic cells (MDCs) were absent from malignant ascites. MDCs derived *in vitro* suppressed angiogenesis *in vivo* through production of interleukin 12. Thus, the tumor may attract PDCs to augment angiogenesis while excluding MDCs to prevent angiogenesis inhibition, demonstrating a novel mechanism for modulating tumor neovascularization. Because dendritic cells (DCs) have long been known to affect tumor immunity, our data also implicate DCs in regulation of tumor neoangiogenesis, suggesting a novel role of DCs in tumor pathology.

### Introduction

Angiogenesis is essential for both primary and metastatic tumor growth. Work to date suggests that vascular endothelial growth factor (VEGF) plays a central role in tumor angiogenesis. However, blood vessel formation is complex and regulated by many factors. For example,  $\beta_3$  and  $\beta_5$  integrins were thought to support angiogenesis based on *in vitro* work. However, recent *in vivo* experiments failed to show support for these molecules in angiogenesis *in vivo* (1, 2). Furthermore, the proangiogenic molecule basic fibroblast growth factor was found to be positively related to the prolonged survival of cancer patients (3). Importantly, early human clinical cancer treatment trials with antiangiogenic molecules have only demonstrated modest benefits (4–6). More strikingly, recent reports (7, 8) suggest that angiogenesis inhibitors (or antagonists) alone, by depriving tumors of oxygen, could have an unintended effect: promotion of tumor metastasis. These results reflect our growing understanding of the complexity of the tumor angiogenic process and metastasis process. Dendritic cells (DCs) prime naïve T cells and thereby activate antigen-specific immunity. The two principal human DC subtypes are MDCs (DC1) and plasmacytoid dendritic cells [PDCs (DC2; Ref. 9)]. MDCs expressing tumor antigens have been used in human clinical trials to

induce significant clinical responses against some tumors (10). Although much work has focused on the relevance of DCs to tumor immunity, there are no reports regarding how DCs influence tumor angiogenesis. Ovarian carcinomas have a poor prognosis, often associated with multifocal intraperitoneal dissemination accompanied by intense neovascularization. Because immune factors are known to modulate blood vessel formation in some settings (11), we hypothesized that specific DC subsets might differentially affect tumor neovascularization.

### Materials and Methods

**Human Subjects.** We studied patients with International Federation of Gynecology and Obstetrics stage III or IV ovarian epithelial carcinomas. All patients gave written, informed consent. The study was approved by the local institutional review board. No patients received prior specific cancer treatments.

**Plasmacytoid Dendritic Cells.** We collected peripheral blood mononuclear cells and ovarian tumor ascites aseptically, harvested cells by centrifugation over a Ficoll-Hypaque density gradient (Amersham), and cryopreserved them at  $-86^{\circ}\text{C}$  until use. CD3-, CD14-, CD16-, CD19-, and CD56-expressing cells were depleted using paramagnetic beads (Miltenyi, Auburn, CA), and blood PDCs or tumor ascites PDCs were sorted by flow cytometry gating on  $\text{CD4}^+\text{CD123}^+\text{CD11c}^-$  cells. Cell populations were  $\geq 99\%$  pure by flow cytometry.

**Myeloid Dendritic Cells.** MDCs were differentiated from  $\text{CD14}^+$  tumor ascites cells with granulocyte macrophage colony-stimulating factor plus interleukin (IL)-4 as described previously (12, 13).

**Activation of Dendritic Cells and Detection of Dendritic Cell-Derived Cytokines.** Tumor-associated PDCs or MDCs were activated for 24 h with CD40 ligand (CD40L) stimulation (200 ng/ml; Immunex) or without stimulation. In some cases, the culture plates were precoated with growth factor-reduced Matrigel (BD Bioscience, Bedford, MA). Dendritic cells were collected for *in vivo* Matrigel assay. Culture supernatants were collected for detecting cytokines with commercial enzyme-linked immunosorbent assay kits (all from R&D Systems, Minneapolis, MN).

***In vivo* Matrigel Assay.** NOD.SCID mice (6–8 weeks old; The Jackson Laboratory, Bar Harbor, ME) were inoculated with growth factor-reduced Matrigel Matrix (BD Bioscience) bearing fresh or CD40L-activated tumor-associated PDCs or MDCs and/or the indicated cytokines and/or mouse anti-human antibody. Recombinant human VEGF, fibroblast growth factor (FGF), tumor necrosis factor (TNF)- $\alpha$ , IL-8 (all at 10 ng/ml) were from R&D Systems. Mouse antihuman TNF- $\alpha$  (clone 1825; IgG1), mouse antihuman-IL-8 antibody (clone 6217; IgG1), and mouse antihuman IL-12 antibody [clone 24910; IgG1 (500 ng/ml each)] were from R&D Systems. After 12 days, we isolated the Matrigel plugs. Matrigel plugs were subjected to immunohistochemistry with anti-von Willebrand factor antibody (polyclonal antibody; 1:10 dilution; DAKO, Carpinteria, CA). Microvessel density was analyzed (14) and quantified with ImagePro Plus software (Media Cybernetics, Silver Spring, MD) and expressed as a mean percentage of microvessel surface area by confocal Leica microscope. Hemoglobin (Hb) content in Matrigel plugs was detected with a commercial kit (Sigma, St. Louis, MO).

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## Results

**Tumor-Associated Plasmacytoid Dendritic Cells Induce Angiogenesis *In vivo*.** To study the role of tumor-associated PDCs in angiogenesis, we purified tumor-associated PDCs from malignant ascites as we described previously (15). We tested the *in vivo* angiogenic effects of freshly isolated primary tumor-associated PDCs in Matrigel (16, 17). We observed significant neovascularization in

Matrigel plugs bearing  $10^6$  primary tumor PDCs ( $P < 0.0001$ , compared with PBS). Activated tumor PDCs ( $10^5$  or  $10^6$ ) also induced *in vivo* neovascularization ( $P < 0.0001$ , compared with PBS). Interestingly,  $10^6$  activated tumor PDCs were significantly more efficient in inducing neoangiogenesis than  $10^6$  primary tumor PDCs ( $P < 0.0001$ ), indicating the enhanced angiogenic capacity of activated tumor PDCs. Furthermore,  $10^6$  activated tumor PDCs induced

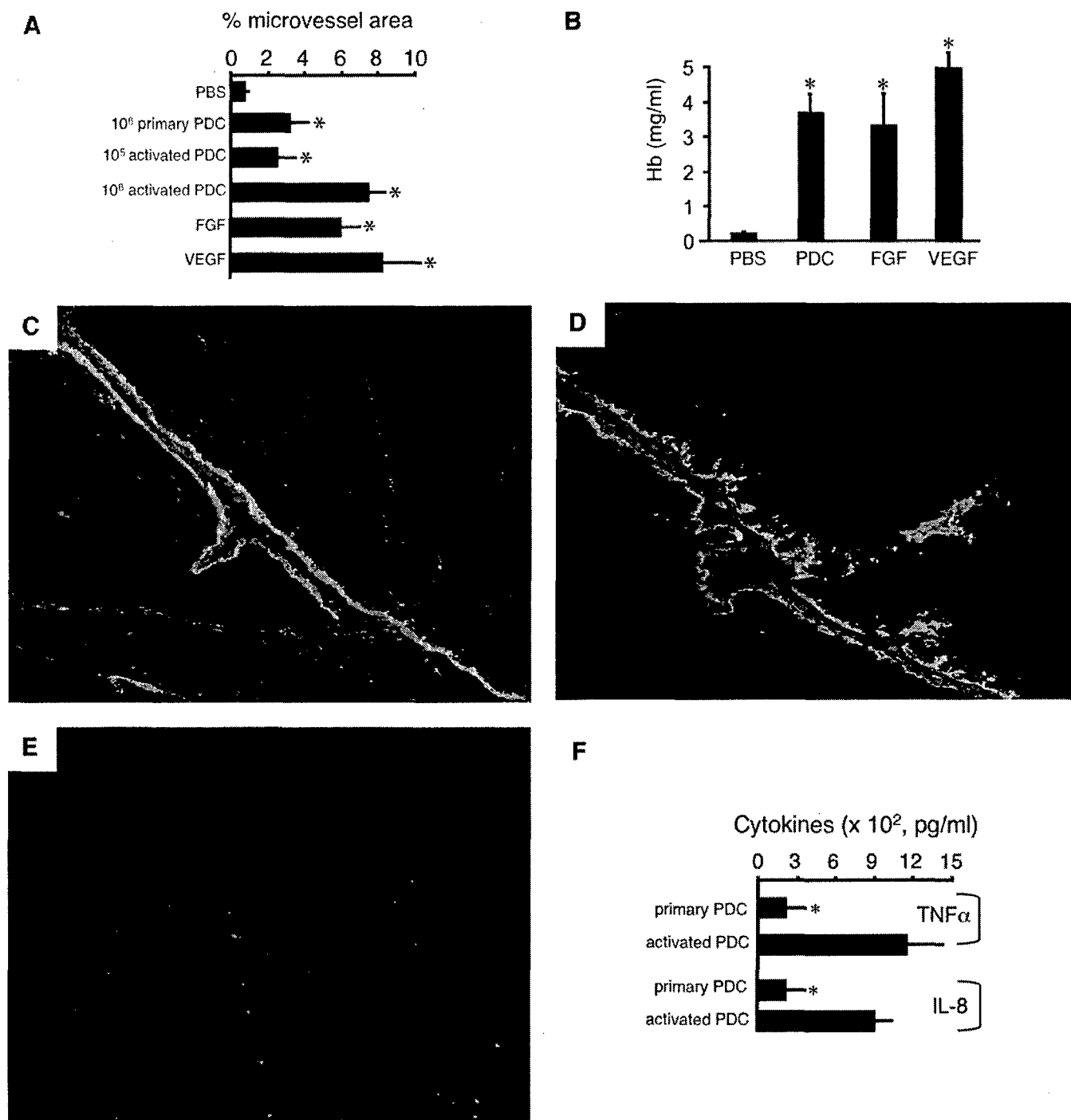


Fig. 1. Tumor PDCs induced angiogenesis *in vivo*. NOD.SCID mice were inoculated with Matrigel plugs bearing tumor-associated PDCs and/or the indicated reagents. *A*, day 12 Matrigel plugs were subjected to immunohistochemistry with anti-von Willebrand factor antibody. Microvessel density was analyzed and expressed as a mean percentage of microvessel surface area (\*,  $P < 0.0001$  for all, compared with PBS). *B*, Hb content in Matrigel plugs was detected (\*,  $P < 0.0001$  for all, compared with PBS). *C–E*, histological analysis showed vascular channel formation and tortuous neovessels in Matrigel plugs with (*C*) tumor-associated PDCs, (*D*) FGF, and (*E*) PBS. Green, von Willebrand factor; red, Topro. *F*, tumor-associated PDCs produced IL-8 and TNF- $\alpha$  (\*,  $P < 0.001$  for all, primary versus activated PDCs). *G*, tumor-associated PDC-derived IL-8 and TNF- $\alpha$  induced angiogenesis *in vivo* (\*,  $P < 0.01$  for all, compared with PDCs alone). *B*, *C*, *F*, and *G*,  $10^6$  PDCs. *A–E* and *G*, 7–10 mice/group.

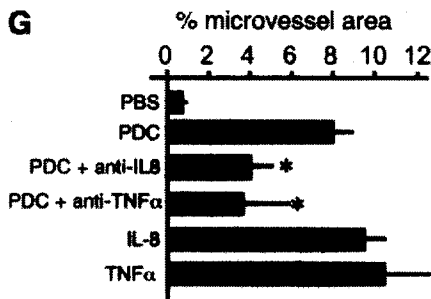


Fig. 1 Continued

more neoangiogenesis than  $10^5$  activated tumor PDCs ( $P < 0.0001$ ), indicating a dose-dependent angiogenic induction. As positive controls, the angiogenic cytokines VEGF and FGF also induced strong angiogenesis *in vivo* ( $P < 0.0001$ , compared with PBS; Fig. 1A).

In our preliminary experiment, Spearman correlation coefficients were computed to assess the relationship between microvessel surfaces and Hb content in Matrigel plugs bearing different concentrations of VEGF (1–50 ng/ml). We showed that the percentage of microvessel surfaces highly correlated with Hb content in Matrigel plugs (Ref. 18; Spearman's  $\rho = 0.32$ ;  $P = 0.0097$ ). Therefore, as an alternative and confirmatory technique, we detected high levels of Hb content in Matrigel plugs containing  $10^6$  activated tumor PDCs, FGF, and VEGF ( $P < 0.0001$  for all, compared with PBS; Fig. 1B). Histological analysis showed vascular channel formation and tortuous neovessels in Matrigel plugs bearing tumor PDCs (Fig. 1C) or FGF (Fig. 1D), but not PBS (Fig. 1E). These data demonstrate that tumor PDCs directly induced angiogenesis *in vivo*.

**Tumor-Associated Plasmacytoid Dendritic Cells Produce Tumor Necrosis Factor  $\alpha$  and Interleukin 8.** We further explored the potential angiogenic mechanisms of tumor-associated PDCs. Strikingly, tumor-associated PDCs spontaneously produced high levels of the angiogenic cytokines TNF- $\alpha$  and IL-8 (Fig. 1F). Consistent with the induced *in vivo* neoangiogenesis, activated tumor PDCs ( $10^6$ /ml) produced significantly more TNF- $\alpha$  and IL-8 ( $n = 6$ ;  $P < 0.001$  for primary PDCs *versus* activated PDCs; Fig. 1F). Tumor PDCs produced undetectable VEGF and IL-12 (data not shown). Furthermore, when tumor PDCs were placed in the plates precoated with growth factor-reduced Matrigel, we detected the identical amount of TNF- $\alpha$  and IL-8, suggesting that the *in vivo*-used growth factor-reduced Matrigel has no direct effects on PDC-derived cytokine production.

**Tumor-Associated Plasmacytoid Dendritic Cell-Derived Tumor Necrosis Factor  $\alpha$  and Interleukin 8 Are Angiogenic.** We hypothesized that tumor PDC-derived TNF- $\alpha$  or IL-8 mediated PDC-driven angiogenesis. Consistent with this hypothesis, anti-TNF- $\alpha$  or anti-IL-8 antibody significantly decreased activated tumor PDC ( $10^6$ )-mediated angiogenesis *in vivo* ( $P < 0.01$ , compared with PDCs alone; Fig. 1G). In confirmation, recombinant TNF- $\alpha$  and IL-8 induced significant angiogenesis *in vivo* ( $P < 0.0001$  for both, compared with PBS; Fig. 1G). Thus, tumor PDCs induce angiogenesis at least in part through TNF- $\alpha$  and IL-8 production *in vivo*.

**Tumor-Associated Myeloid Dendritic Cells Inhibit Angiogenesis *In vivo*.** We observed no significant numbers of MDCs in malignant ascites in patients with ovarian cancers (15). To evaluate the potential angiogenic role of tumor-associated MDCs *in vivo*, we differentiated tumor-associated MDCs from malignant ascites macrophages as we described previously (12, 13, 15). Tumor-associated MDCs did not induce angiogenesis *in vivo*, even after activation with CD40L. Recombinant human VEGF induced significant angiogenesis *in vivo* ( $P < 0.0001$ , compared with PBS; Fig. 2A). Interestingly,

VEGF mediated-angiogenesis was significantly reduced by  $10^6$  or  $5 \times 10^6$  CD40L-activated tumor MDCs ( $P < 0.01$ , compared with VEGF alone; Fig. 2A), indicating that MDCs suppress angiogenesis *in vivo*. Tumor-associated MDCs ( $5 \times 10^6$ ) were more efficient in suppressing VEGF-mediated angiogenesis than  $10^6$  tumor-associated MDCs, indicating a dose-dependent angiogenic reduction ( $P < 0.05$ ,  $5 \times 10^6$  *versus*  $10^6$  MDCs; Fig. 2A). The Hb content (Fig. 2B) was also consistent with the percentage of microvessel quantification (Fig. 2A).

Additionally, we detected high levels of VEGF by enzyme-linked immunosorbent assay in malignant ascites ( $1440 \pm 739$  pg/ml;  $n = 12$ ) and primary ovarian carcinoma tumor cell cultures ( $340 \pm 139$  pg/ml VEGF produced by  $10^6$  tumor cells/ml in 48 h). Thus, tumor-associated MDCs suppressed angiogenesis induced by tumor-derived VEGF.

**Tumor-Associated Myeloid Dendritic Cell-Derived Interleukin 12 Suppresses Angiogenesis *In vivo*.** We further studied the angiogenic suppressive factor derived from MDCs. Anti-IL-12 antibody blocked the suppressive effects of MDCs on angiogenesis ( $P < 0.01$ ,  $10^6$  MDCs + VEGF *versus*  $10^6$  MDCs + VEGF + anti-IL-12), suggesting that MDC-derived IL-12 was the angiogenic suppressive factor (Fig. 2). In support of this, recombinant IL-12 inhibited VEGF-mediated angiogenesis ( $P < 0.01$ , VEGF *versus* VEGF + IL-12; Fig. 2). The data further support that MDCs are able directly to suppress angiogenesis *in vivo* through IL-12 production. In confirmation, we showed that after CD40L activation, tumor-associated MDCs produced significant IL-12 ( $1830 \pm 250$  pg/ml;  $n = 8$ ). We observed the identical amount of IL-12 produced by MDCs cultured with the plates precoated with growth factor-reduced Matrigel. It indicates that the growth factor-reduced Matrigel has no detectable effects on MDC cytokine production.

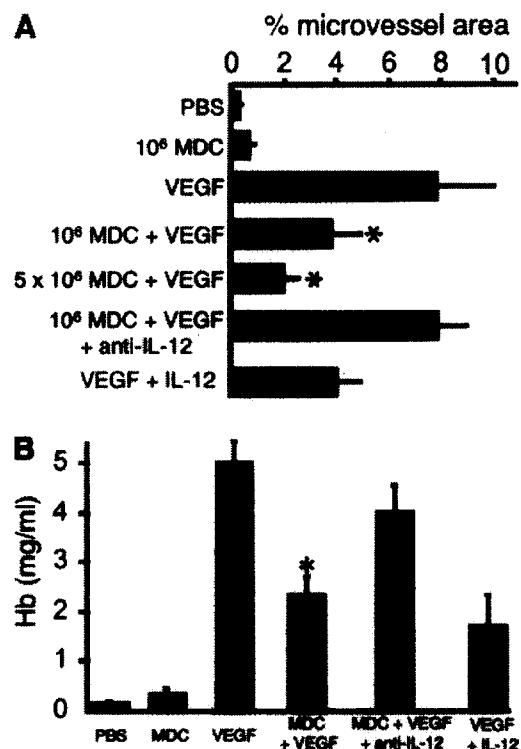


Fig. 2. Tumor MDCs suppress angiogenesis *in vivo*. NOD.SCID mice were inoculated with Matrigel plugs bearing activated tumor-associated MDCs plus the indicated reagents. Day 12 Matrigel plugs were removed to study neovascularization as described in the Fig. 1 legend. A, MDCs suppress VEGF-induced angiogenesis through IL-12. B, microvessel surface data are correlated with the Hb contents in Matrigel plugs. A total of  $10^6$  MDCs were used in B. A and B, 7–10 mice/group. \*,  $P < 0.001$ , MDCs + VEGF *versus* VEGF.

## Discussion

Tumor angiogenesis is essential for the growth of primary and metastatic tumors. The angiogenic process requires the coordinated activities of multiple factors and cell types (4, 11). Tumor angiogenesis occurs when the effect of angiogenic stimulatory factors outweighs that of inhibitory factors (11). Although vascular endothelial cells and their released angiogenic molecules have been studied extensively, the potential angiogenic roles of immune cells, particularly those of dendritic cells, in the tumor microenvironment have not been well defined.

There are substantial numbers of functional PDCs (but not MDCs) in tumor ascites in patients with ovarian carcinomas (15). MDCs produce significant amounts of IL-12 and induce high levels of T-cell interferon (IFN)- $\gamma$  (15). IL-12 and IFN- $\gamma$  are potent angiogenic inhibitory cytokines (11). We hypothesize that MDCs may suppress tumor angiogenesis *in vivo* through these cytokines. Lack of MDCs in the tumor microenvironment may be orchestrated by the tumor to minimize the angiogenesis-inhibiting effects of these MDCs. Consistent with our hypothesis, we showed here that MDCs significantly suppressed angiogenesis *in vivo*. MDC-derived IL-12 is a critical factor inhibiting tumor angiogenesis. These data may help explain the beneficial effects of MDCs in reducing tumor burden even when they do not bear tumor-associated antigens.

Cells resembling PDCs were observed in histological sections of lymphoid tumors, granulomas, and multicentric Castleman's disease (19–21). Apart from our recent report (15), however, functional PDCs have never been recovered from pathological tissues for study. There are no reports regarding whether PDCs have effects on tumor angiogenesis. After virus infection, PDCs produce a large amount of type I IFN [IFN- $\alpha$ , IFN- $\beta$ , and IFN- $\omega$  (15, 22–24)]. IFN- $\alpha$  and IFN- $\beta$  are potent tumor angiogenic inhibitory cytokines. Numerous functional PDCs accumulate in tumor ascites; however, type I IFNs are undetectable in tumor ascites (15). In contrast to MDCs, T-cell signal CD40L stimulates little or undetectable IL-12 production by human PDCs (25, 26). We recently demonstrated that PDCs, but not MDCs, accumulate in the tumor environment and that tumor PDCs inhibit antitumor immunity (15). We now show that tumor PDCs produce high levels of the angiogenic cytokines TNF- $\alpha$  and IL-8 and induce potent neovascularization *in vivo*. Thus, tumors manipulate DC distribution and alter DC function to support tumor angiogenesis. MDCs suppress tumor angiogenesis. PDCs enhance tumor angiogenesis.

In summary, our data demonstrate a novel role for DCs in human cancer. The DC system is relevant to tumor angiogenesis: MDCs inhibit tumor angiogenesis; and tumor-associated PDCs enhance tumor angiogenesis. Maximal vascularization of tumors may thus require the simultaneous accumulation of PDCs and the absence of MDCs, as observed in ovarian tumors. Blocking PDC-mediated neovascularization in tumors may be a novel strategy to treat human cancers.

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# IMMUNOSUPPRESSIVE NETWORKS IN THE TUMOUR ENVIRONMENT AND THEIR THERAPEUTIC RELEVANCE

Weiping Zou

**Abstract** | It is well known that many tumours are potentially immunogenic, as corroborated by the presence of tumour-specific immune responses *in vivo*. Nonetheless, spontaneous clearance of established tumours by endogenous immune mechanisms is rare. Therefore, the focus of most cancer immunotherapies is to supplement essential immunogenic elements to boost tumour-specific immunity. Why then has tumour immunotherapy resulted in a generally poor clinical efficiency? The reason might lie in the increasingly documented fact that tumours develop diverse strategies that escape tumour-specific immunity.

## TOLERANCE

A state in which the immune system does not mount effective immune responses against specific antigens.

## ANTIGEN-PRESENTING CELLS

Cells that uptake, process and present antigen to other immune cells to initiate and activate immune responses. Monocytes, macrophages, dendritic cells and B cells are antigen-presenting cells. Dendritic cells are the most potent of these.

The immune system can discriminate between a range of stimuli, allowing some to provoke immune responses, which leads to immunity, and preventing others from doing so, which leads to TOLERANCE. To this end, the immune system is highly organized and orchestrated at various levels (molecular, cellular and systemic).

In the context of tumour immunology, tumour immunity or tumour immune tolerance means the success or failure, respectively, of the immune system to reject a tumour. The tumour microenvironment, which is composed of immune cells, tumour cells, stromal cells and the extracellular matrix, is a main battleground during the neoplastic process, fostering proliferation, survival and migration of tumour cells. Not only can tumours survive and disseminate, but, more importantly, they can mimic some of the signalling pathways of the immune system to propagate conditions that favour tumour immune tolerance — 'tolerizing' conditions — and so escape tumour immunity. The focus of this review is the interaction between tumours and the immune system in the human tumour environment, showing how tumour immune tolerance starts gradually and locally, progresses and finally spreads to the whole organism. I suggest that tumour immune tolerance is initiated and maintained by a constitutive interaction between tumours and the host immune system, and controlled by variable modifications to the

immune response present in the tumour environment. Finally, I will summarize how tumour immune tolerance predominates and outweighs the effective immune response in patients with cancer, and how we might be able to subvert tumour tolerance to achieve a successful tumour immunotherapy.

## Overview

In 1863, Rudolf Virchow observed leukocyte infiltration of tumours and for the first time suggested a possible functional relationship between inflammatory infiltrates and malignant growth. In 1957, Burnet and Thomas postulated the existence of tumour immunosurveillance: the immunological resistance of the host against the development of cancer. This concept has been challenged for decades because of the lack of direct experimental evidence<sup>1,2</sup>. Between the mid-1970s and 1990s, experimental data from several groups seemed to support the existence of tumour surveillance, and by 2003 accumulating new evidence proved that the immune system is able to recognize and defeat tumours<sup>3-7</sup>. However, spontaneous tumour eradication is rare. It was originally thought that the inefficiency of tumour-associated antigen (TAA)-specific immunity was due to intrinsic causes: tumours simply did not present enough TAA; ANTIGEN-PRESENTING CELLS (APCs) did not have sufficient stimulatory capacity; or there were

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# Summary

- The pathological interactions between cancer cells and host immune cells in the tumour microenvironment create an immunosuppressive network that promotes tumour growth, protects the tumour from immune attack and attenuates immunotherapeutic efficacy.
- Poor tumour-associated antigen (TAA)-specific immunity is not simply due to a passive process whereby adaptive immunity is shielded from detecting TAAs. There is an active process of 'tolerization' taking place in the tumour microenvironment.
- Tumour tolerization is the result of imbalances in the tumour microenvironment, including alterations in antigen-presenting cell subsets, co-stimulatory and co-inhibitory molecule alterations and altered ratios of effector T cells and regulatory T cells.
- Human tumorigenesis is a slow process that can occur over several years and in this respect is similar to chronic infection. The lack of an acute phase in the course of tumorigenesis might profoundly shape T-cell immune responses, including the quality of antigen release, T-cell priming and activation.
- Current immunotherapies often target patients with advanced-stage tumours, which have high levels of inflammatory molecules, cytokines, chemokines, tumour-infiltrating T cells, dendritic cells and macrophages. It is arguable whether we need to incorporate more of these components into tumour treatments.
- Immune tolerization is predominant in the immune system in patients with advanced-stage tumours. It is time to consider combinatorial tumour therapies, including those that subvert the immune-tolerizing conditions within the tumour.

not enough EFFECTOR T CELLS or effector cytokines. On this basis, attempts were made to bolster TAA-specific immunity through administration of stimulatory cytokines (interleukin-2 (IL-2), IL-12 or interferon- $\alpha$  (IFN $\alpha$ )) or tumour-associated antigens (peptides), or by using optimal APCs (dendritic-cell (DC) vaccines)<sup>4-13</sup>. In a different approach, TAA-specific effector T cells from cancer patients were expanded *ex vivo* followed by adoptive transfer<sup>6,9,14-16</sup>. These approaches were met with some success both in mouse models and in early clinical trials in humans. Therefore, the principle that experimentally induced TAA-specific immunity is a potentially efficacious approach to treat established human tumours is evident.

However, more recent work from our laboratory and others demonstrates that poor TAA-specific immunity is not due to a passive process whereby adaptive immunity is shielded from detecting TAA. On the contrary, there is an active process of 'tolerization' taking place in the tumour microenvironment. So if the goal is to produce active immunity, either naturally or experimentally, the tolerizing conditions must first be overcome. In BOX 1 several novel anticancer therapeutic strategies that attempt to reverse these tolerizing conditions are shown. Tolerization within the tumour microenvironment is a complex process and involves interactions between several different cell types.

## TAAs and TAA-specific priming

CD8<sup>+</sup> effector T cells have a central role in the elimination of tumours. These cells recognize 8-10 amino acid long peptides buried in the antigen-presenting groove of major histocompatibility complex class I molecules<sup>17</sup>. Recent studies of infection indicate that antigens are presented in the draining lymph nodes a few hours after the subcutaneous injection of a pathogen<sup>18,19</sup>. Activation of

naive CD8<sup>+</sup> T cells is a relatively rapid process — 2.5 hours of antigen exposure are sufficient to induce clonal expansion and differentiation of CD8<sup>+</sup> T cells *in vitro*<sup>20,21</sup>, and 24 hours of pathogen presence are sufficient for the induction of protective CD8<sup>+</sup> T-cell responses *in vivo*<sup>22</sup>. Therefore, CD8<sup>+</sup> T-cell priming takes place at a very early stage of infection — the acute infection phase. This rapid process seems to be crucial for effective expansion of antigen-specific T-cells, followed by contraction and formation of memory T cells. However, the T-cell priming process is poorly understood in the context of TAA-specific T-cell immunity. Human tumorigenesis is a slow process that, like chronic infection, might occur over several years. Acute infection might 'warm up' the immune system for subsequent contest. For instance, acute infection might be crucial for systemic antigen distribution and efficient induction of innate immunity including NATURAL KILLER CELL (NK cell) activation. It is well known that activated NK cells stimulate the maturation of DCs and facilitate adaptive antitumour immunity. Indeed, NK cells link innate immunity and adaptive immunity<sup>23</sup>. Therefore, it could be speculated that induction of potent TAA-specific T-cell immunity needs an efficient early activation of NK cells, as is the case during infection. However, the lack of an acute phase in the course of tumorigenesis might lead to a poor early activation of NK cells, and thereafter profoundly reshape T-cell immune responses against the tumour.

Although it is controversial, it is suggested that before the development of systemic metastasis (including lymphoid metastasis), the antigenic cancer cells (expressing TAAs) are embedded in the solid tumour. The stroma of the tumour prevents the efficient release of TAAs<sup>24</sup>, which are ignored in the conventional central priming sites — the draining lymph nodes<sup>25,26</sup>. In the later stages of tumour development, TAAs are thought to be efficiently released, which induces the protective immune system to mount an effective response<sup>27,28</sup>. However, tolerizing mechanisms already abound in the tumour microenvironment by this stage, disabling the functions of APCs and effector T cells. In support of this, recent studies have shown that effector CD8<sup>+</sup> T-cell responses to common melanoma epitopes are generally weak, localized, and occur mostly in patients with advanced metastatic disease<sup>27,28</sup>. Taken together, these data indicate that the quality of tumour-associated T-cell priming and the kinetics and temporal release of TAAs actively participate to elicit an effective tumour immune response.

Additionally, TAA-priming might happen in the tumour microenvironment to some degree, where naive T cells and APCs can be found. Indeed, extranodal TAA-specific priming has been reported<sup>29,30</sup>. However, the tumour is a 'false' lymphoid organ, and T-cell priming in the tumour microenvironment has at least two basic defects. The APCs present in the tumour environment are either dysfunctional or induce T-cell tolerance, as discussed below, and the infrastructure of tumours is different from that of lymph nodes. Tumour tissues show increased

### EFFECTOR T CELLS

T cells that exert a cytolytic function following engagement of their T-cell antigen receptor on target cells. CTLs express the co-receptor CD8 and recognize antigenic peptides that are presented by human leukocyte antigen class I molecules.

### NATURAL KILLER CELLS

Natural killer cells are a type of cytotoxic lymphocyte that can be distinguished from CD8<sup>+</sup> T cells by their lack of rearrangement of T-cell receptor genes. They have abundant granule-containing cytoplasm, induce target-cell death through direct contact or by cytokine production, and confer innate immunity.

**Box 1 | Tumour immunotherapy**

Improving the tumour-associated immune response can be achieved by either boosting components of the immune system that produce an effective immune response or by inhibiting components that suppress the immune response.

The treatments indicated in the table below are based on the hypothesis that immune elements are missing or not sufficient in patients with tumours. The strategy would be to supplement whatever is missing or not sufficient, thereby boosting tumour-associated antigen (TAA)-specific immunity, and improving patient outcome.

Immune elements	Enhancing 'the enhancers'
T cells	Injection of cytotoxic T lymphocytes
Dendritic cells (DCs)	DC vaccination
Natural killer cells	Injection of activated natural killer cells
TAA	Peptide, TAA-vector vaccination
Effector cytokines	Administration of interleukin-2, interferon- $\alpha$ and interleukin-12

The treatments indicated in table below are based on the hypothesis that immune tolerance is dominant in tumour patients. The strategy would be to block these suppressive mechanisms, followed by immune boosting as detailed above with the aim of recovering and boosting TAA-specific immunity and improving patient outcome.

Suppressive elements	Inhibiting 'the inhibitors'
Regulatory T ( $T_{Reg}$ ) cells	Blocking $T_{Reg}$ cell function (for example, using denileukin difitox to kills $T_{Reg}$ cells)
Suppressive, dysfunctional DCs	Blocking suppressive pathways (for example, targeting cells that express B7-H1, B7-H4 or indoleamine-2,3-deoxygenase)
Self antigen or immodominant	Fostering antigen release by targeting the stroma antigen and/or improving antigen tumour priming
Suppressive cytokines	Blocking potential common cytokine signalling pathway (for example, targeting signal transducer and activator of transcription 3 and suppressor of cytokine signalling 1)

**MYELOID DENDRITIC CELLS**  
A subset of dendritic cells that are lineage-negative CD11c<sup>+</sup>HLA-DR<sup>+</sup> mononuclear cells with a monocytoïd appearance. Human myeloid dendritic cells might differentiate from myeloid precursors (for example, monocytes, macrophages and CD11c<sup>+</sup> precursors).

**PLASMACYTOID DENDRITIC CELLS**  
A subset of dendritic cells that are lineage negative HLA-DR<sup>+</sup>CD11c<sup>+</sup> mononuclear cells with a microscopic appearance similar to plasmablasts. Plasmacytoid dendritic cells are the main producers of type I IFN.

interstitial fluid pressure and contain defective blood vessels. There is fibrosis and contraction of the interstitial matrix. Many tumours lack lymph vessels. These structural defects would potentially contribute to the poor or dysfunctional T-cell priming in the tumour microenvironment. Fu *et al.* recently showed that forced expression of the tumour-necrosis factor (TNF) superfamily member LIGHT in the tumour environment induces priming of naive T cells and leads to the rejection of established, highly progressive tumours at local and distal sites in mice. These data indicate that an efficient T-cell priming in the tumour microenvironment is possible<sup>29</sup>. Tumour immune vaccination has for a long time been focused on fostering T-cell central priming in draining lymph nodes. As peripheral TAA-specific T-cell priming is possible, although it is technically challenging, one could postulate that another alternative would be to restructure the tumour into a real functional 'lymphoid organ' and to engineer 'quality' peripheral TAA-specific priming (for example, T-cell priming within the tumour microenvironment), which would overcome the potential TAA ignorance in the draining lymph nodes of a tumour.

**Human APCs**

APCs include DCs, monocytes/macrophages and B lymphocytes. DCs are a heterogeneous group of APCs that display differences in anatomic localization, cell-surface phenotype, and function<sup>31,32</sup>. However, all DCs have several features in common. First, DCs are differentiated from CD34<sup>+</sup> bone-marrow stem cells. Second, immature DCs are thought to localize in the periphery and are specialized in the uptake and processing of antigens. In turn, mature DCs are found in lymphoid organs, where they interact with antigen-specific T cells and initiate immune responses. Third, immature DCs present distinct chemokine receptors compared with mature DCs, and these regulate their transport into tissue sites. Human DCs are traditionally divided into two main populations: MYELOID DCs and PLASMACYTOID DCs<sup>10,32,33</sup>.

DCs were initially thought to be exclusively immunogenic, actively inducing or upregulating immune responses. However, recent advances demonstrated that DCs possess dual functions, and can also show regulatory (suppressive) activity. DCs are able to actively downregulate an immune response or to induce immune tolerance by influencing the activity of other cell types. The biology of DCs and DC-based clinical trials has been extensively reviewed elsewhere<sup>8,10,13,31,34</sup>. This review is limited to discussing the recent advances in the understanding of DCs present in the tumour microenvironment, including immature/partially differentiated myeloid DCs, B7-H1<sup>+</sup> (also known as PD-L1<sup>+</sup>) myeloid DCs, INDOLEAMINE-2,3-DEOXYGENASE (IDO)<sup>+</sup> myeloid DCs, tumour-associated plasmacytoid DCs, and vascular (CD11c<sup>+</sup>CD45<sup>+</sup>) DCs. It seems that these DCs within the tumour microenvironment possess a regulatory function. Although it is possible that professional 'regulatory' DCs exist, I favour the view that a regulatory function is not the intrinsic nature of a distinct DC subset<sup>35–39</sup>. DCs recruited to the tumour microenvironment undergo changes that endow them with regulatory functions favourable for the tumour. Therefore, it can be hypothesized that through a different 'instruction' this process could be reversed.

**Tumour environmental myeloid DCs**

Myeloid DCs arise from the same progenitor cells that also give rise to monocytes and macrophages, and express, at least in humans, cell-surface markers characteristic of the myeloid lineage<sup>31</sup>. Mature myeloid DCs induce a strong T<sub>H</sub>1 (T<sub>H</sub>1)-type immune response and are considered potent inducers of TAA-specific immunity. However, the presence of functional immunogenic (mature) myeloid DCs is rare in human ovarian tumours<sup>35</sup>, breast tumours<sup>40,41</sup>, prostate cancer<sup>42</sup> and renal-cell carcinomas<sup>43</sup>. Various factors such as a defective DC recruitment, differentiation, maturation and survival could be the cause. Abundant evidence documents that the differentiation and maturation of myeloid DCs is profoundly suppressed by factors present in the tumour microenvironment. Cancer cells can be major producers of vascular endothelial growth factor (VEGF)<sup>44,45</sup>, causing high VEGF levels in the tumour

# B7-H1

A recently defined B7 family member that is found to be expressed in human epithelial tumours, and can be induced in antigen-presenting cells and in non-lymphoid organs. PD-1 is the identified receptor. Experimental evidence indicates the existence of an unidentified receptor for B7-H1. B7-H1 can mediate an inhibitory role as well as stimulatory role in T-cell-mediated immune responses.

# INDOLEAMINE 2,3-DIOXYGENASE

An intracellular haeme-containing enzyme that catalyses oxidative catabolism of tryptophan.

# T HELPER 1 CELLS AND T HELPER 2 CELLS

Two functionally defined CD4<sup>+</sup> T-cell subsets. T helper 1 (T<sub>H</sub>1) cells predominantly produce interferon- $\gamma$ , and support cellular immunity. T<sub>H</sub>2 cells predominantly produce interleukin-4, and support humoral immunity.

# TUMOUR ASCITES

An accumulated fluid in the peritoneum due to cancer. In some cancers, the ascites fluid contains viable tumour cells, immune cells and soluble factors. Tumour ascites is an accessible tumour environment for research.

# REGULATORY T CELLS

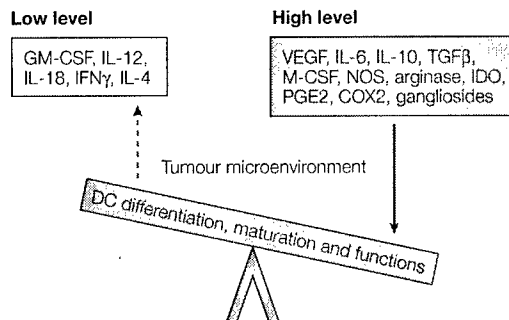
A T-cell population that can functionally suppress an immune response by influencing the activity of another cell type. There might exist several phenotypically distinct regulatory T cells. The classic ones are CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> T cells.

# NOD/SCID MICE

Mice that do not have T cells or B cells. Tumour cells can be grown in these mice without rejection.

# B7-H4

A newly defined B7 family member that is found to be expressed in human ovarian epithelial tumours, non-lymphoid organs and antigen-presenting cells within the tumour microenvironment. The receptor remains to be identified. The B7-H4 fusion protein can mediate a profound inhibitory role in T-cell-mediated immune responses.



**Figure 1 | An aberrant tumour microenvironmental molecule pattern and dendritic cells.** The tumour microenvironment is characterized by a lack of molecules promoting dendritic cell (DC) differentiation and function (granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4)) and cytokines inducing T helper 1 (T<sub>H</sub>1)-type responses (IL-12, IL-18 and interferon- $\gamma$  (IFN $\gamma$ )), but is abundant in molecules suppressing DC differentiation and function (vascular endothelial growth factor (VEGF), IL-6, IL-10, transforming growth factor- $\beta$  (TGF $\beta$ ), macrophage colony-stimulating factor (M-CSF), arginase, indoleamine-2,3-deoxygenase (IDO), prostaglandin E2 (PGE2), cyclooxygenase-2 (COX2) and nitric-oxide synthase 2 (NOS2)). These suppressive molecules are largely produced by tumour cells, macrophages and stromal cells in the tumour microenvironment. This aberrant molecule pattern profoundly affects tumour-specific immunity.

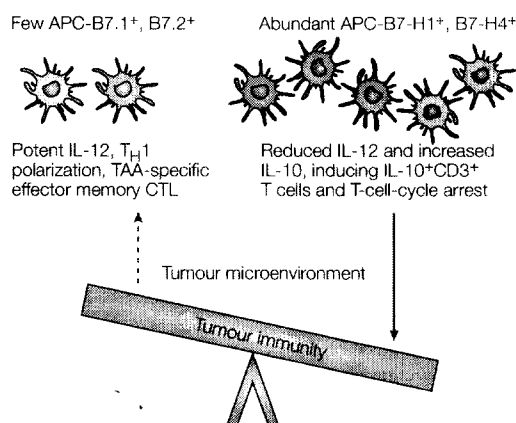
microenvironment. VEGF was the first tumour-derived molecule reported to suppress DC differentiation and maturation<sup>38</sup>. Subsequently, IL-6 and macrophage colony-stimulating factor (M-CSF) derived from tumour cells and macrophages from the tumour microenvironment were shown to switch DC differentiation towards macrophage differentiation by upregulating the expression of the M-CSF receptor in monocytes<sup>46</sup>. High levels of IL-6 and C-reactive protein (CRP) can be found in the peripheral blood and malignant ascites of patients with ovarian cancer<sup>44,45,47,48</sup>. Notably, IL-6 can promote B-cell differentiation and has been suggested to block the suppressive activity of regulatory T cells (T<sub>Reg</sub> cells)<sup>49</sup>. Moreover, a range of human tumours express high levels of cyclooxygenase-2 (COX2)<sup>50-52</sup>. COX2 promotes prostaglandin E2 (PGE2) production in the tumour environment. PGE2 in turn suppresses DC differentiation and function<sup>53-56</sup>. Additionally, tumour cells, tumour-associated macrophages and T<sub>Reg</sub> cells often produce IL-10 and transforming growth factor- $\beta$  (TGF $\beta$ ), which also suppress DC maturation and function. Human neuroblastoma, melanoma and many other tumours express high levels of gangliosides, which also suppress human DC differentiation<sup>57,58</sup>. Strikingly, DC differentiation cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4, as well as the T<sub>H</sub>1-type cytokines IL-12 and IFN $\gamma$ , are rare in the human tumour microenvironment, at least in human ovarian cancer. Abundance of VEGF, IL-6, M-CSF, TGF $\beta$ , IL-10, COX2, PGE2, gangliosides and other suppressive molecules versus negligible GM-CSF, IL-4,

IL-12, and IFN $\gamma$  causes an aberrant cytokine pattern in the tumour environment (FIG. 1)<sup>47,48</sup>. This cytokine imbalance in the tumour microenvironment blocks DC differentiation and maturation. Whereas functional mature myeloid DCs can induce potent TAA-specific immunity *in vivo*<sup>59,60</sup>, immature or partially differentiated myeloid DCs induce either suppressive T<sub>Reg</sub> cells<sup>61,62</sup> or T-cell unresponsiveness<sup>63</sup>. The induced suppressive T<sub>Reg</sub> cells home to draining lymph nodes and, in turn, systemically disable TAA-specific immunity. Therefore, immature or partially differentiated myeloid DCs can function as regulatory DCs and are an important component of the immunosuppressive networks in the tumour microenvironment.

# Tumour environmental B7-H1<sup>+</sup> myeloid DCs

As well as inhibiting DC differentiation and maturation, tumour factors present in the local environment profoundly affect the functional capacity of DCs to activate T-cell TAA-specific immunity, by triggering myeloid DC B7-H1 expression<sup>36</sup>. B7.1 and B7.2 (also known as CD80 and CD86, respectively) are B7 family members with co-stimulatory functions for T-cell activation. B7-H1 is a recently identified B7 family member. There is approximately 25% homology between B7.1, B7.2 and B7-H1 (REF. 64). Factors within the tumour microenvironment stimulate B7-H1 expression in myeloid DCs present in ovarian tumours and their draining lymph nodes. A significant fraction of tumour-associated T cells are T<sub>Reg</sub> cells<sup>65</sup>, which express PD-1, the ligand for B7-H1 (REF. 66). Tumour-associated T cells can then, through reverse signalling through B7-H1, suppress IL-12 production by myeloid DCs, and therefore reduce their immunogenicity<sup>36</sup>. Blocking B7-H1 enhances myeloid-DC-mediated T-cell activation<sup>36,67</sup> and reduces the growth of a transplanted human ovarian carcinoma in non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice adoptively transferred with autologous human TAA-specific T cells<sup>36</sup>. Induction of B7-H1 on myeloid DCs by factors within the tumour microenvironment is a novel mechanism for immune evasion by a tumour<sup>36</sup>. On the other hand, expression of B7-H1 on human cancers such as ovarian cancer, lung cancer, melanoma, glioblastoma and squamous-cell carcinoma<sup>68</sup> also contributes to immune evasion by inducing apoptosis of effector T cells<sup>68</sup>, so facilitating tumour growth<sup>69</sup>. PD-1 is one of the ligands for B7-H1. PD-1 blockade by genetic manipulation (producing PD-1<sup>-/-</sup> cells) or antibody treatment efficiently inhibits mouse B16 melanoma and CT26 colon cancer dissemination and metastasis accompanied with increased effector T-cell number and enhanced function<sup>70,71</sup>. In summary, the significant influence of tumour environmental B7-H1 and PD-1 on the interaction between T cells, tumour cells and APCs constitutes a novel target for tumour immunotherapy.

The complex interactions between B7-H1, its receptors and other ligands, as well as between B7-H1-expressing cells and effector T cells, still remain largely unknown<sup>66</sup>. B7-H4 (also known as B7X or B7S1) is a



**Figure 2 | Imbalance of co-stimulatory and co-inhibitory molecules on antigen-presenting cells within the tumour microenvironment.** Antigen-presenting cells (APCs) within the tumour microenvironment express a low level of co-stimulatory molecules (B7.1 and B7.2) and a high level of co-inhibitory molecules (B7-H1 and B7-H4). Molecules within the tumour microenvironment selectively stimulate the expression of co-inhibitory molecules and inhibit co-stimulatory molecules on APCs. These co-inhibitory molecules disable APC immunogenicity and induce APCs to become regulatory APCs with diverse suppressive mechanisms. CTL, cytotoxic T lymphocyte; IL, interleukin; TAA, tumour-associated antigen;  $T_H1$ , T helper 1.

recently discovered member of the B7 family. The ligand (or ligands) of B7-H4 remains unidentified. B7-H4 was shown to be a negative regulator of T-cell responses *in vitro* by inhibiting T-cell proliferation, cell-cycle progression and cytokine production<sup>66,72–74</sup>. Antigen-specific T-cell responses were impaired in mice treated with a B7-H4-immunoglobulin fusion protein<sup>72</sup> and we have found that ovarian epithelial tumour cells and tumour-infiltrating APCs including macrophages express high levels of B7-H4. Interestingly, B7-H4<sup>+</sup> tumour-infiltrating macrophages potentially suppress TAA-specific T-cell immunity (W.Z. *et al.*, unpublished observations). Collectively, cytokines within the tumour microenvironment might selectively suppress the expression of co-stimulatory molecules (B7.1 and B7.2) and trigger the expression of co-inhibitory molecules (B7-H1 and B7-H4) in APCs, so contributing to immune evasion by the tumour (FIG. 2).

Factors such as IL-10 within the tumour microenvironment selectively modulate the expression of B7 family members so as to tilt the balance towards immune suppression: co-inhibitory molecules (B7-H1 and B7-H4) are upregulated and co-stimulatory molecules (B7.1 and B7.2) are downregulated. Considering that B7-H1 can have an immune-stimulatory function<sup>75,76</sup>, it remains to be defined why it mediates an immunosuppressive effect within the tumour microenvironment. Nonetheless, an imbalance between co-stimulatory molecules (B7.1 and B7.2) and co-inhibitory molecules (B7-H1 and B7-H4) is created in the tumour environment (FIG. 2), and spreads into tumour-draining lymph nodes, which

'instructs' immunogenic APCs to become regulatory APCs. Co-inhibitory molecules become the tumour's 'mask and weapon', initially to avoid immune attack, and then to reduce T-cell priming and defeat the invasion of effector T cells.

### Tumour environmental IDO<sup>+</sup> myeloid DCs

IDO expression has been documented in human and murine myeloid DCs<sup>77</sup>. IDO catalyses the oxidative catabolism of tryptophan, an amino acid essential for T-cell proliferation and differentiation. IDO<sup>+</sup> DCs reduce access to free tryptophan and so block the cell-cycle progression of T-cells. This, in turn, prevents the clonal expansion of T cells and promotes T-cell death by apoptosis, ANERGY or immune deviation<sup>77</sup>. IDO<sup>+</sup> DCs can be generated *in vitro* from monocyte-derived DCs, and this has also been found *in vivo*, in breast tumour tissue and tumour-draining lymph nodes in patients with melanoma and patients with breast, colon, lung and pancreatic cancers<sup>77</sup>. In light of findings by Munn, Mellor and colleagues, it is conceivable that IDO<sup>+</sup> DCs contribute to tumour-mediated immunosuppression<sup>77</sup>. In line with the role of IDO<sup>+</sup> DCs, transfection of IDO renders tumour cell lines immunosuppressive *in vitro* and *in vivo*. Systemic treatment of mice with an inhibitor of IDO — 1-methyltryptophan — significantly delays tumour growth in mouse models<sup>78</sup>. Notably, IDO expression by murine DCs is upregulated by the fusion protein CYTOTOXIC T-LYMPHOCYTE-ASSOCIATED PROTEIN 4 (CTLA4)–immunoglobulin<sup>79</sup>, indicating that CTLA4-expressing cells, such as tumour-associated CTLA4<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>  $T_{reg}$  cells (see below)<sup>65</sup>, induce IDO expression in DCs within the tumour microenvironment, and effectively convert them into regulatory DCs. It is unclear at present whether B7-H1- or B7-H4-expressing myeloid DCs are phenotypically identical to IDO-expressing myeloid DCs in the human tumour microenvironment. Nonetheless, the presence of IDO<sup>+</sup> myeloid DCs in tumour-draining lymph nodes and within the tumour microenvironment would further tip the balance of tumour microenvironmental DC subsets in favour of tolerizing conditions (FIG. 3).

### Tumour environmental plasmacytoid DCs

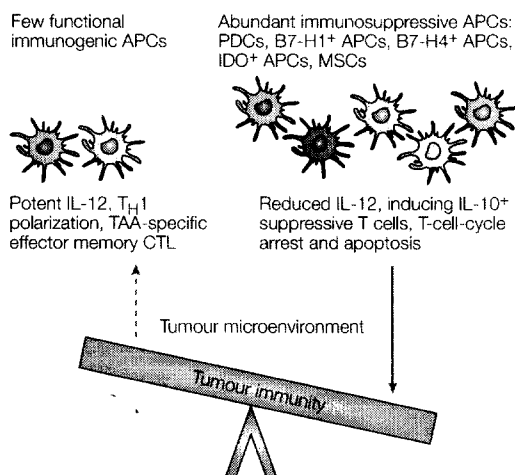
Functional plasmacytoid DCs are found in the local tumour environment of patients with ovarian cancer<sup>85</sup>, melanoma<sup>80</sup> and head and neck squamous-cell carcinoma<sup>81</sup>. Tumour cells produce the chemokine ligand CXCL12 (also known as stromal-cell-derived factor 1) and plasmacytoid DCs express CXCR4, the receptor for CXCL12. Tumour-derived CXCL12 mediates trafficking of plasmacytoid DCs into the tumour (FIG. 4)<sup>35</sup>. CXCL12 protects tumour plasmacytoid DCs from apoptosis<sup>35</sup>. *In vitro*, activated peripheral-blood plasmacytoid DCs stimulate tumour-specific T-cell IFN $\gamma$  production in patients with melanoma<sup>80</sup>. Interestingly, plasmacytoid DCs within the tumour microenvironment show reduced expression of toll-like receptor 9 (TLR9)<sup>81</sup>, which is the most specific TLR pathway for inducing IFN $\alpha$ . IFN $\alpha$  not only triggers innate immunity, such as activating NK cells, but also promotes adaptive

#### ANERGY

A state in which lymphocytes can not respond to antigen-specific and non-specific stimulation.

#### CYTOTOXIC T-LYMPHOCYTE-ASSOCIATED PROTEIN 4

Following engagement by B7.1 or B7.2 on antigen-presenting cells, cytotoxic T-lymphocyte-associated protein 4 (CTLA4) signalling in activated T cells induces cell-cycle arrest, and reduces cytokine production, and diminishes T-cell responses. CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells constitutively express CTLA4.



**Figure 3 | Imbalances in antigen-presenting-cell subsets and tumour immunity.** The tumour microenvironment lacks functionally mature myeloid dendritic cells (DCs) capable of inducing tumour-specific T-cell immunity, whereas it is abundant in immunosuppressive antigen-presenting cell (APC) subsets, including partially differentiated myeloid DCs, B7-H1<sup>+</sup> DCs, indoleamine-2,3-deoxygenase (IDO)<sup>+</sup> DCs, plasmacytoid DCs (PDCs) and myeloid suppressor cells (MSCs). These suppressive APC subsets induce arrest of the T-cell cycle, suppressive T cells and T-cell apoptosis. This APC-subset imbalance favours the formation of immune tolerance. CTL, cytotoxic T lymphocyte; IL, interleukin; TAA, tumour-associated antigen T<sub>H</sub>1, T helper 1.

T<sub>H</sub>1-type T-cell responses. This indicates that plasmacytoid DCs can be phenotypically and functionally modulated in the tumour microenvironment. In support of this, plasmacytoid DCs within the tumour microenvironment induce significant IL-10 production by T cells that suppresses myeloid-DC-induced TAA-specific T-cell effector functions<sup>35</sup>. Our recent unpublished data reveal that tumour plasmacytoid DCs induce IL-10<sup>+</sup>CCR7<sup>+</sup>CD8<sup>+</sup> T cells to home to draining lymph nodes, and suppress TAA-specific central priming (W.Z. *et al.*, unpublished observations). The fact that ALLOGENEIC plasmacytoid DCs are able to induce CD4<sup>+</sup> (REF. 82) and CD8<sup>+</sup> (REF. 83) suppressive T<sub>Reg</sub> cells supports these data. A large amount of plasmacytoid DCs, but not functional mature myeloid DCs, accumulate in the tumour microenvironment<sup>35</sup>. We suggest that the imbalanced distribution of APC subsets in the tumour microenvironment further contributes to immune evasion by a tumour (FIG. 3). We are currently exploring the underlying mechanisms by which tumour plasmacytoid DCs induce suppressive T cells.

#### Tumour vascular DCs

Functional myeloid DCs are able to produce IL-12 and induce potent cytokine production of IFN $\gamma$  and IL-10. All three molecules are strong suppressors of tumour angiogenesis<sup>45</sup>. Human myeloid DCs, but not plasmacytoid DCs, are the major producers of IL-12. It is not surprising then that myeloid DCs suppress tumour

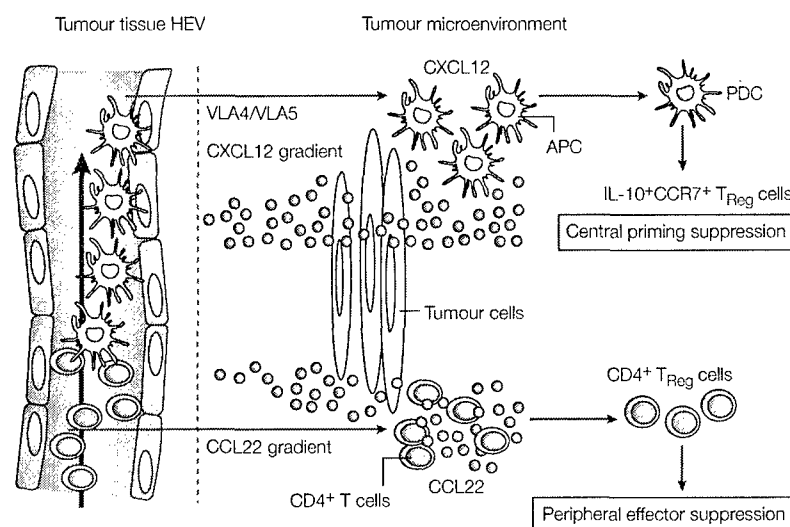
neovascularization<sup>37</sup>, which is essential for tumour growth and metastasis<sup>45</sup>. Strikingly, tumour environments seem to lack angiogenesis-inhibitory myeloid DCs, but present abundant angiogenesis-stimulatory DCs, such as plasmacytoid DCs<sup>37</sup> and vascular DCs<sup>84</sup>. Tumour-derived CXCL12 attracts and protects plasmacytoid DCs in the tumour microenvironment and these cells can induce vascularization by spontaneously producing TNF $\alpha$  and IL-8 (REF. 37). Coukos *et al.* demonstrated that vascular DCs are recruited by  $\beta$ -defensin, and induce vasculogenesis under the influence of VEGF in mice. Vascular DCs are also found in ovarian tumours<sup>84</sup>. Therefore, DCs are relevant not only in tumour immunopathogenesis, but also in tumour vascularization. Optimal vascularization of tumours might require the simultaneous accumulation of vascular DCs and the absence of anti-angiogenic myeloid DCs, as is observed in ovarian tumours. Imbalance between vascular-inhibitory DCs and vascular-stimulatory DCs in the tumour microenvironment also contributes to tumour immunopathogenesis (FIG. 5). The suppression of neovascularization of tumours mediated by vascular DCs might be a novel strategy to treat human cancer.

#### Tumour myeloid suppressor cells

Murine myeloid suppressor cells (MSCs) represent a phenotypically heterogeneous cell population that includes immature and mature myeloid cells, activated granulocytes, macrophages, as well as cells expressing markers of immature DCs. MSCs can function as APCs. Murine MSCs are distinct from suppressive lymphocytes and can be phenotypically identified as CD11b<sup>+</sup>Gr-1<sup>+</sup>CD31<sup>+</sup> (REFS 85,86). Human MSCs were found in patients with head and neck squamous-cell carcinoma, non-small-cell lung carcinoma, and metastatic adenocarcinomas of the pancreas, colon and breast<sup>87,88</sup>. However, there are no specific markers described for human MSCs<sup>88</sup>. MSCs infiltrate into tumour-draining lymph nodes to inhibit priming of naive T cells, and also into tumour tissues<sup>89-92</sup> (FIG. 3). Murine MSCs use two enzymes involved in L-arginine metabolism to control T-cell responses: inducible nitric-oxide synthase 2 (NOS2), which generates nitric oxide (NO) and arginase-1 (ARG1), which depletes the milieu of L-arginine. Induction of NOS2 is mainly controlled by IFN $\gamma$  and TNF $\alpha$ . After inducing NOS2, MSCs subsequently release NO. NO acts at the level of IL-2 receptor signalling, blocking the phosphorylation and activation of several signalling molecules, which induces T-cell apoptosis<sup>85,86,90</sup>. ARG1 is often induced by cytokines within the tumour microenvironment, such as TGF $\beta$  and IL-10. L-arginine is essential for T-cell function, including the optimal use of IL-2 and the development of a T-cell memory phenotype<sup>91,92</sup>. Depletion of nutrients is a strategy used throughout nature to control the growth of organisms competing for the same biological niche. Unfortunately, this strategy is incidentally available in tumours and defeats the immune response.

#### ALLOGENEIC

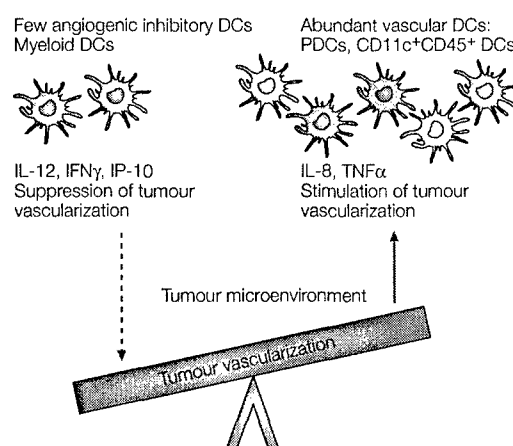
Allogeneic tissues or cells are genetically different from the host and can elicit an immune response when transplanted into immune-competent hosts.



**Figure 4 | Specific recruitment and tumour immune evasion.** Tumour-derived chemokine CXCL12 mediates the trafficking of plasmacytoid dendritic cells (PDCs) into tumours. Tumour PDCs induce interleukin-10 (IL-10)<sup>+</sup>CCR7<sup>+</sup> suppressive CD8<sup>+</sup> T cells, which traffic into tumour-draining lymph nodes and inhibit tumour-specific central priming. The chemokine CCL22, derived from tumour cells and associated macrophages, mediates CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> regulatory T (T<sub>Reg</sub>) cell trafficking into tumours. CD4<sup>+</sup>T<sub>Reg</sub> cells predominantly inhibit effector T-cell function in the tumour environment. HEV, high endothelial venule.

### Tumour APCs and immunotherapy

It is evident that there is an imbalance of APC subsets in the tumour environment (FIG. 3). Lack of functional mature DCs and abundance of suppressive DCs significantly reduce the TAA-specific T-cell priming in draining lymph nodes, as well as the TAA-specific effector immunity in the tumour microenvironment. An aberrant molecule network further contributes to this detrimental situation. Absence of DANGER SIGNALS, including inflammatory cytokines, molecular and cellular T-cell



**Figure 5 | Imbalances in antigen-presenting-cell subsets and tumour vascularization.** The tumour microenvironment lacks functional angiogenesis-inhibitory myeloid dendritic cells (DCs), whereas it is abundant in angiogenesis-stimulatory macrophages and DC subsets, including plasmacytoid DCs (PDCs) and CD11c<sup>+</sup>CD45<sup>+</sup> vascular DCs, are present. The imbalance of antigen-presenting cell subsets in the tumour microenvironment promotes tumour vascularization.

#### DANGER SIGNALS

Triggers that are associated with host-cell damage. These 'danger signals' induce the activation of antigen-presenting cells. The danger signals could be intracellular components that are released when cells are damaged, such as DNA and proteins, or inflammatory molecules and cytokines, such as interferon- $\gamma$ .

activation signals in the tumour microenvironment, has been considered the main cause of poor tumour immunity<sup>2,93,94</sup>. Therefore, incorporation of danger signals as a strategy to boost tumour immunity has been suggested<sup>2,93,94</sup>. However, high levels of IL-6, COX2, CRP and other danger signals are observed in many stage I tumours, including ovarian cancer<sup>47,48</sup>, in accordance with the idea that cancer can be accompanied by chronic inflammatory reactions<sup>95,96</sup>. There are at least three danger signals associated with tumours: hypoxic stress, metabolic stress and immune-attack stress. As these signals are mostly present in late tumour stages, it is possible that danger signals might be more limited in microscopic tumours before clinical diagnosis. This might also account for the poor immunogenicity of micrometastases.

Notably, current immunotherapies often target patients with advanced-stage tumours, manifesting high levels of danger signals in the tumour microenvironment, including inflammatory molecules, cytokines, chemokines, tumour-infiltrating T cells, DCs and macrophages. As discussed above, insufficient arousal of fully functional effector T cells as well as lack of fully immunogenic APCs are evident in tumour microenvironment in these patients. However, tolerization has been established and is predominant in these patients. It is therefore arguable whether we need to further enhance these danger signals in tumours. Indeed, non-steroidal anti-inflammatory agents, including aspirin, sulindac and NS398, are able to reduce tumour formation in mouse models and in patients at high risk for colon carcinoma<sup>97–100</sup>. COX2 inhibitors are able to suppress human tumour growth in mice<sup>101,102</sup>, and enhance the efficiency of radiotherapy<sup>103</sup>. More importantly, one cannot definitively rule out that danger signals or passively supplemented immune elements alone could enhance immune stress in tumours<sup>6</sup>, and strengthen the aberrant cytokine network in some circumstance. For instance, IFN $\gamma$  is an effector cytokine released by TAA-specific T cells. However, IFN $\gamma$  strongly stimulates B7-H1 (REFS 68,104), IDO<sup>77</sup> and arginase<sup>85,86,90</sup> expression on APCs. Such APCs could induce suppressive tumour immunity. So it is not a surprise that *Ifn $\gamma$* <sup>-/-</sup> mice manifest autoimmune diseases. Furthermore, certain strategies of tumour vaccination induce IL-10-expressing CD4<sup>+</sup>CD25<sup>+</sup> T cells in mice<sup>105</sup> and patients with melanoma<sup>106</sup>. Therefore, as suggested previously<sup>4,6</sup>, current immunotherapy strategies including cytokine administration, DC vaccination and adoptive T-cell transfusion might possibly contain elements that counteract therapeutic efficiency. The occasional marked clinical efficacy of immunotherapies might represent cases in which immune tolerance is not dominant.

As mentioned above, tumour immunotherapies including DC vaccines often target late-stage tumours with the aim of boosting TAA immunity. Fully activated DCs are less sensitive to suppression and able to rescue suboptimal activated T cells<sup>8,10,13,31,107</sup>. However, it remains to be defined whether fully activated DCs alone would subvert the suppressive capacity of T<sub>Reg</sub> cells and of regulatory (or dysfunctional) APCs in tumour-bearing patients. For instance, in patients with ovarian cancer,

Table 1 | Subversion of 'tolerizing' conditions

Targets	Suggested potential treatments	References
Tumour microenvironmental plasmacytoid DCs and tumour	AMD3100 blocks CXCR4 signal <i>in vivo</i> ; AMD3100 could reduce the trafficking of plasmacytoid DCs into the tumour by blocking the CXCR4 signal; AMD3100 might also suppress tumour vascularization/metastasis	35,44,109,110
	CpG nucleotides activate plasmacytoid DCs and trigger production of type I IFN by plasmacytoid DCs	35,112,113
B7-H1 <sup>+</sup> myeloid DCs and tumours	Neutralizing anti-B7-H1	36,66,68,70,71
B7-H4 <sup>+</sup> macrophages and tumours	Neutralizing anti-B7-H4	64,72-74
	Specific B7-H4-blocking oligonucleotides	W.Z. <i>et al.</i> , unpublished observations
IDO <sup>+</sup> MSCs and tumours	IDO inhibitor (1-methyltryptophan)	39,77,78
Suppressive cytokines IL-6 and IL-10	Blocking the common pathway STAT3 or SOCS1	141,142
Arginase <sup>+</sup> MSCs	Arginase inhibitor ( <i>N</i> -hydroxy-nor-L-arginine)	92
T <sub>Reg</sub> cells	Denileukin difitox (Ontak) kills CD25 <sup>+</sup> leukaemia cells, indicating that it might kill CD25 <sup>+</sup> T <sub>Reg</sub> cells	140
	Anti-CD25 depletes CD25 <sup>+</sup> T <sub>Reg</sub> cells	125,126
	Anti-CTLA4 blocks CTLA4 signal	127,136-139,143
	Anti-CCL22 reduces trafficking of T <sub>Reg</sub> cells into the tumour	65

DC, dendritic cell; IDO, indoleamine-2,3-deoxygenase; IFN, interferon; IL, interleukin; MSC, myeloid suppressor cell; SOCS1, suppressor of cytokine signalling 1; STAT3, signal transducer and activator of transcription 3; T<sub>Reg</sub> cell, regulatory T cell.

repetitive stimulation with fully matured myeloid DCs is not able to recover T-cell dysfunction mediated by tumour-associated plasmacytoid DCs (Zou *et al.*, unpublished observations). Even after a successful surgical intervention, many patients will present with residual disease. Tolerizing conditions will have already been established in these patients and will remain present after surgery. Adoptively transferred DCs will face identical tolerizing situations as the 'original' DCs did in the tumour microenvironment and could partially explain the poor clinical efficiency of DC vaccinations. Furthermore, fully activated DCs would possibly expand and activate CD4<sup>+</sup>CD25<sup>+</sup> T<sub>Reg</sub> cells<sup>108</sup>. Tackling these tolerizing conditions might improve the efficacy of DC vaccines. AMD3100 is a CXCR4 antagonist, and has been used in human clinical trials for treatment of human immunodeficiency virus (HIV) infection<sup>109</sup>. CXCR4–CXCL12 signals are implicated in the trafficking of plasmacytoid DCs into tumours<sup>35</sup> and CXCL12–CXCR4 signals are further involved in tumour vascularization<sup>44</sup> and metastasis<sup>110</sup>. It is tempting to speculate that administration of AMD3100 or other CXCR4–CXCL12 signalling antagonists could block the trafficking of plasmacytoid DCs into tumours, interrupt the interaction between tumours and plasmacytoid DCs, and in turn disable tumour plasmacytoid-DC-mediated immunosuppression and vascularization. CXCL12–CXCR4 antagonists would further suppress CXCL12–CXCR4-mediated tumour neoangiogenesis<sup>44</sup> and metastasis<sup>110</sup>. In addition, activated plasmacytoid DCs in the tumour microenvironment remain as a chief source of type I IFN<sup>35</sup>, potentially bridging innate and adaptive immunity. IFN $\alpha$  derived from plasmacytoid

DCs might induce maturation of myeloid DCs and activate NK cells in the tumour microenvironment, and also provide important T-cell survival signals<sup>111</sup>. IFN $\alpha$  is also a potent inhibitor of tumour angiogenesis<sup>45</sup>. Given the potential benefit of activating plasmacytoid DCs, the use of oligonucleotides as an adjuvant to activate plasmacytoid DCs has been suggested to treat various diseases including human tumours<sup>112,113</sup>. CpG OLIGONUCLEOTIDE 7909 is now in Phase I/II clinical trial in patients with basal-cell carcinoma or with melanoma<sup>113</sup>.

The immunosuppressive activity of IDO-expressing APCs or tumour cells can be inhibited by the IDO inhibitor 1-methyltryptophan. The *D*-isomer of 1-methyltryptophan is the candidate for human clinical trials (D. Munn, personal communication). The immunosuppressive activity of arginase-expressing MSCs might be inhibited by an arginase inhibitor, *N*-hydroxy-nor-L-arginine (Nor-NOHA). The injection of Nor-NOHA and Nor-NOHA plus L-arginine in tumour-bearing mice significantly inhibits tumour growth in a dose-dependent manner<sup>92</sup>. As most of human epithelial tumours and APCs express B7-H1 and B7-H4 (REFS 36,64,68,72), these molecules represent additional attractive new targets for cancer immunotherapy. As *in vitro* and *in vivo* experiments in mice demonstrate that blocking B7-H1 (REFS 36,67,69,70) and B7-H4 (W.Z., unpublished observations) pathways improves TAA-specific T-cell immunity, it would be therapeutically meaningful to develop the related signal pathway blocking molecules for human clinical trials. These and other potential novel immunotherapeutic strategies are listed in TABLE 1.

**CpG OLIGONUCLEOTIDES**  
Synthetic oligodeoxynucleotides (CpG-ODNs) that contain CpG motifs that are similar to those found in bacterial DNA that can stimulate an immune response. These CpG-ODNs have various potential therapeutic uses.

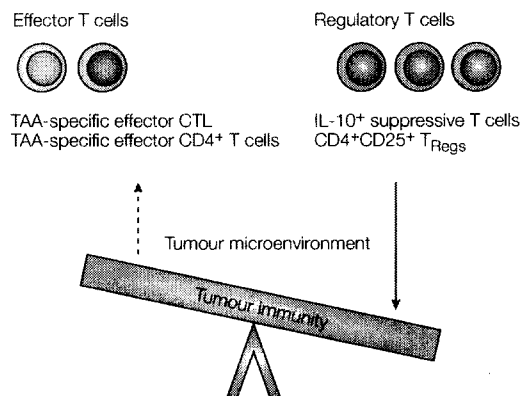
### Tumour $T_{Reg}$ cells

A  $T_{Reg}$  cell is functionally defined as a T cell that inhibits an immune response by influencing the activity of another cell type<sup>114</sup>. The most well-defined  $T_{Reg}$  cells are  $CD4^+CD25^+$  T cells. The field of  $T_{Reg}$  cells is a rapidly evolving area of investigation and has been extensively reviewed in the context of basic biology, autoimmune diseases, organ transplantation and infectious diseases<sup>115–117</sup>.

Although  $T_{Reg}$  cells were originally identified in mice, a population with identical phenotypic and functional properties has been identified in humans.  $CD4^+$   $T_{Reg}$  cell subsets include naturally occurring  $CD4^+CD25^+$   $T_{Reg}$  cells as well as peripherally induced  $CD4^+$   $T_{Reg}$  cells, or IL-10-expressing  $T_{Reg}$  cells<sup>115,116,118–121</sup>. As discussed above, IL-10 and TGF $\beta$  produced by ovarian tumours favour the induction and differentiation of  $T_{Reg}$  cells. Dysfunctional myeloid DCs<sup>36</sup> and tumour-conditioned plasmacytoid DCs<sup>35</sup> would directly contribute to  $T_{Reg}$  cell induction in the tumour microenvironment. Tumour-bearing patients have increased numbers of both peripherally circulating and tumour-associated  $CD4^+CD25^+$   $T_{Reg}$  cells<sup>122–124</sup>. These  $T_{Reg}$  cells are functional, as shown by inhibition of non-specific T-cell activation *in vitro*.

Experimental administration of antibodies against CD25 (REFS 125,126) and of antibodies against CTLA4 (REFS 127,128) in tumour-bearing mice improves immune-mediated tumour clearance and enhances the response to immune-based therapy. These original studies indicate that  $CD25^+$  and CTLA4<sup>+</sup> cells might be detrimental to tumour immunity in mice. Notably, although mouse  $CD4^+CD25^+$  T cells constitutively express CTLA4 (REFS 129,130), the link has not been demonstrated between anti-CTLA4 treatment and the reduction of the number or function of  $CD4^+CD25^+$  T cells. Furthermore, depletion of  $CD25^+$  cells and CTLA4 blockade were shown to act synergistically<sup>131</sup>, indicating that CTLA4 blockade directly promotes T-cell function. On the other hand, anti-CD25 administration in humans also prevents transplant rejection, indicating that the CD25 marker does not discriminate between effector T cells and  $T_{Reg}$  cells<sup>116</sup>, suggesting that anti-CD25 might deplete  $CD25^+$  effector T cells.

The role of  $CD4^+CD25^+$   $T_{Reg}$  cells was recently investigated in mice bearing B16 melanoma cells. The study clearly demonstrates that  $CD4^+$   $T_{Reg}$  cells are the major regulators of concomitant tumour immunity against this weakly immunogenic tumour<sup>132</sup>. In addition, our recent study in humans provides the first direct *in vitro* and *in vivo* evidence that human  $T_{Reg}$  cells have an important immunopathological role in human cancer by 'lowering' the endogenous TAA-specific T-cell immunity, contributing to tumour growth. Accumulation of  $T_{Reg}$  cells in the tumour tilts another tumour environmental balance — the balance between  $T_{Reg}$  cells and effector T cells — in favour of immunosuppression (FIG. 6). Tumour  $T_{Reg}$  content is linked with a striking reduction of patient survival, providing evidence linking  $T_{Reg}$  cells to the pathogenesis of human cancer<sup>65</sup>.



**Figure 6 | T-cell-subset imbalance and tumour-specific immunity.** Tumour-associated antigen (TAA)-specific effector T cells are able to kill tumour cells. Regulatory T ( $T_{Reg}$ ) cells reduce the killing capacity of TAA-specific T cells. A large amount of  $T_{Reg}$  cells infiltrating into tumours tilts the balance of T-cell subsets within the tumour microenvironment in favour of 'tolerizing' conditions.

How do  $T_{Reg}$  cells traffic to the tumour microenvironment? Ovarian tumour  $T_{Reg}$  cells express functional CCR4, the receptor for CCL22, and migrate towards it in response to CCL22 in the tumour microenvironment, which is produced by tumour cells and associated macrophages (FIG. 4). Homeostatic and tumour  $T_{Reg}$  cells express similar amounts of the lymphoid-homing molecules CCR7 and CD62L. However, whether these molecules have roles in the lymphoid homing by  $T_{Reg}$  cells is unknown. Furthermore, trafficking and distribution of  $T_{Reg}$  cells could be dynamically variable during tumour progression. One would predict that in the very early phase of tumorigenesis (earlier than clinically defined tumour stage I),  $T_{Reg}$  cells might predominantly localize in draining lymph nodes, whereas in late stages of tumour progression  $T_{Reg}$  cells will predominantly reside in the tumour microenvironment. The former would primarily suppress priming of naive T cells, whereas the latter would mainly inhibit the function of extranodal effector cells.

Under the stress of an immune response it seems likely that tumours that recruit and activate host  $T_{Reg}$  cells are more likely to survive —  $T_{Reg}$  cells would disarm T-cell effector mechanisms. Infectious pathogens have evolved the same strategy to escape immune attack and maintain a chronic infection<sup>133</sup>. Interestingly, cancer has also been considered a chronic inflammatory disease<sup>134</sup>. Not only do 15% of cancers worldwide have a direct infectious origin<sup>135</sup>, but also many human tumours are related to chronic irritation and inflammation. This hypothesis is of the utmost importance when designing tumour immunotherapy.

### Tumour $T_{Reg}$ cells and immunotherapy

Given that  $T_{Reg}$  cells within the tumour microenvironment are a crucial component of the tumour immunosuppressive network, the clinical depletion of

$T_{Reg}$  cells in patients with tumours could become a promising strategy for boosting TAA-specific immunity.

Depletion of CD25<sup>+</sup> cells using an antibody against CD25 resulted in improved tumour rejection in mice<sup>125,126</sup>. CTLA4 attenuates the potency of TAA-specific T-cell immunity and, in mice, administration of an antibody that blocks CTLA4 function inhibited the growth of moderately immunogenic tumours<sup>136</sup>. In addition, suppression of CTLA4 in combination with cancer vaccines or depletion of CD25<sup>+</sup> cells increases the rejection of poorly immunogenic tumours, albeit with evidence of an autoimmune response<sup>131,136,137</sup>. Administration of an inhibitory antibody to CTLA4 in previously vaccinated cancer patients resulted in effective antitumour immunity<sup>138</sup>. The human anti-CTLA4 antibody (MDX-010) in conjunction with peptide vaccination improved tumour immunity and tumour regression in patients with metastatic melanoma<sup>139</sup>. Although blocking CTLA4 can induce significant autoimmune manifestations<sup>138,139</sup>, the studies provide the proof of principle for CTLA4 suppression in treatment of human cancer. Notably, similar to mouse  $T_{Reg}$  cells, human CD4<sup>+</sup>CD25<sup>+</sup>  $T_{Reg}$  cells in the tumour microenvironment constitutively express CTLA4 (REF. 65). However, it has not been demonstrated that treatment with antibodies against CTLA4 reduces the content of CD4<sup>+</sup>CD25<sup>+</sup>  $T_{Reg}$  cells or alters the function  $T_{Reg}$  cells either in mice or in humans. Nonetheless, these studies indicate that the depletion of CD4<sup>+</sup>  $T_{Reg}$  cells or/and a suppression of  $T_{Reg}$  cell function could weaken  $T_{Reg}$ -cell-mediated tolerance.

Denileukin diftitox (Ontak) is a ligand toxin fusion consisting of full-length IL-2 fused to the enzymatically active and translocating domains of diphtheria toxin<sup>140</sup>. This complex is internalized into CD25<sup>+</sup> cells by endocytosis. The ADP-ribosyltransferase activity of diphtheria toxin is cleaved in the endosome and is translocated into the cytosol, where it inhibits protein synthesis, leading to apoptosis<sup>140</sup>. This drug has been approved by the Food and Drug Administration in the United States for treatment of CD4<sup>+</sup>CD25<sup>+</sup> cutaneous T-cell leukaemia/lymphoma. Predictably, denileukin diftitox depletes CD4<sup>+</sup>CD25<sup>+</sup>  $T_{Reg}$  cells, and our and other laboratories are now testing this concept in patients with lung, ovarian or other cancers, where levels of such  $T_{Reg}$  cells are greatly increased<sup>165,122,123</sup>. Therefore, depletion of  $T_{Reg}$  cells

or blockade of  $T_{Reg}$ -cell-specific transport represents a novel means to augment tumour immunity, which might be used in a range of novel therapeutic approaches. Denileukin diftitox represents the first agent that is potentially useful for humans in this regard.

# Future directions

Although 'black and white' signals have been identified in tumour immunity, it is evident from the discussions above that this is an oversimplification and that interactions between tumour cells and immune cells would be best represented by a multitude of colours. Therefore, experimental interpretations are mostly qualitative. It is time to move on to characterize molecules and cells, and their activities and their interactions in a quantitative way, temporally and spatially. In this regard, we need rigorous methods and models to dissect the complicated suppressive *in situ* network, and to make decisions considering all (or most) molecular and cellular components. For instance, scientists have successfully applied mathematical models to define the *in vivo* dynamics of HIV replication, and the *in vivo* turnover of its target — CD4<sup>+</sup> T cells. These findings facilitated the establishment of highly active antiretroviral therapy to treat HIV-infected patients. Similarly, quantitative and dynamic models will be essential to dissect the differentiation, expansion, trafficking, half-life and function of  $T_{Reg}$  cells, as well as the distribution, expression, regulation and interaction of 'inhibitory' (B7-H1, B7-H4, CTLA4 and IDO) and 'stimulatory' (B7.1 and B7.2) molecules. How do all these factors form the immunosuppressive network in the course of tumour development and progression? A better understanding of these kinetic aspects could be used to develop and refine novel immune-boosting strategies.

Finally, after many years of effort, a 'proof of principle' for boosting antitumour immunity in humans has been established. However, it might not be sufficient to passively supplement the essential immune elements, including adoptively transferring tumour antigens, T cells, cytokines and DCs. It is time to consider combinatorial tumour therapies. I suggest a '3S' therapeutic strategy: subversion of tolerizing conditions (S1), supplementation of immune elements (S2), and suppression of tumour angiogenesis and growth (S3).

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#### Competing interests statement

The author declares no competing financial interests.

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